PHARMACOGNOSY, PRELIMINARY PHYTOCHEMICAL AND PHARMACOLOGICAL STUDY OF SPERMACOCE HISPIDA.L

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BY
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UNDER THE GUIDANCE OF
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DECLARATION BY THE CANDIDATE

I declare that this thesis, **"Pharmacognosy, Preliminary, Phytochemical and Pharmacological study of *Spermacoce hispida* L.,"** submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of Dr. Subrahmanya Kumar K. and Co-Guide of Dr. Noorunnisa Begum S. I confirm that no part of the work reported herein has been submitted for a degree or examination at any other university. References, funding, and material obtained from other sources have been duly acknowledged, and no part of this dissertation has been plagiarized.

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CERTIFICATE FROM THESIS SUPERVISOR/S

This is to certify that the work incorporated in this thesis **"Pharmacognosy, Preliminary Phytochemical and Pharmacological study of *Spermacoce hispida* L."** submitted by Dr Raveena AR was carried out under my/our supervision. No part of this thesis has been submitted for a degree or examination at any other university. References, help and material obtained from other sources have been duly acknowledged. I confirm the originality of the work and that there is no plagiarism in any part of the thesis.

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SUMMARY

Spermacoce hispida L., known as "bukah" and "vasukha" in Ayurveda, is a medicinal plant that is used in traditional medicine. It is a plant that is commonly misidentified with other species of the same genus. In the southern part of India, it is used in folklore medicine for reducing body weight. This traditional knowledge is validated in the current study, which includes pharmacological standardisation of the plant, preliminary phytochemical detection, and related pharmacological studies for analysing the effect of *S. hispida* Kashaya on whole body fat in the model organism *Drosophila melanogaster*.

Macroscopic investigations point out specific morphological traits that set it apart from other *Spermacoce* species. Microscopic studies reveal the presence of trichomes, plano-convex-shaped leaves, quadrangular stems, round roots, and calcium oxalate crystals in transverse sections. Histochemical analyses of the stem and root show the presence of polyphenols. Preliminary Phytochemical detection tests, including high-performance thin-layer chromatography (HPTLC), demonstrate the presence of various classes of compounds, notably polyphenols, flavonoids, terpenes, alkaloids, and glycosides, in successive Soxhlet extractions of *S. hispida* using different solvents. In the *Drosophila* model, Kashaya of *S. hispida* did not show a substantial reduction in whole body fat in flies fed on a high fructose diet. The study emphasises the need for further investigations to explore its potential therapeutic benefits.

PERSONAL REFLECTION

During the course of my thesis, I had the opportunity to gain practical experience in various aspects of pharmacognosy, phytochemistry, and experimental biology. In the pharmacognosy lab, one of the essential skills I developed was the identification of plants based on their morphological characteristics. Through macroscopic studies, I learned to distinguish between different plant species and specifically recognise the distinct features of *Spermacoce hispida*. This skill enabled me to accurately collect the plant material needed for my research. Another valuable technique I acquired during my thesis research was the preparation of an herbarium.

Anatomical sectioning of plant tissues was another technique I learned during my time in the pharmacognosy lab. The preparation of thin sections of the root, stem, and leaf samples allows me to study the histological characteristics of *S. hispida* and understand the arrangement of cells, vascular bundles, and crystals within the plant.

In the phytochemistry lab, I developed skills in preliminary phytochemical detection tests. This involved performing qualitative analyses to identify the presence of various classes of compounds, including polyphenols, terpenes, and alkaloids, in *S. hispida* extracts. Additionally, I gained experience in handling high-performance thin-layer chromatography (HPTLC) for the separation and analysis of the chemical constituents present in the plant samples. Lastly, I gained expertise in fly husbandry and conducted TAG assays as part of my experimental biology work.

Overall, my thesis research provided me with a diverse skill set spanning various disciplines. From plant identification and anatomical sectioning in the pharmacognosy lab to phytochemical analysis and fly husbandry in the laboratory of experimental biology.

LIST OF ABBREVIATIONS

Spermacoce hispida	*S. hispida*
Stomata	ST
Abaxial surface	ABE
Adaxial Surface	ADE
Vascular Bundle	VB
Mesophyll	MS
Palisade parenchyma	PP
Spongy parenchyma	SP
Tannin	Ta
Xylem	Xy
Phloem	Ph
Trichome	Tri
Parenchyma	Pa
Cortex	Co
Sclerenchyma	Scl
Triacylglycerol	TAG
Benzoic acid	BA
Orthophosphoric acid	OP
Propionic Acid	PA

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1.INTRODUCTION

Ethnomedicine encompasses a wide range of traditional healing practises, remedies, and beliefs that are deeply rooted in different cultures and passed down through generations. Traditional medicine, as defined by the World Health Organisation (WHO), encompasses a wide range of knowledge, skills, and practises that are rooted in cultural beliefs, ideas, and experiences. It is used for various purposes, including health maintenance, prevention, improvement, diagnosis, and treatment of physical and mental ailments. The use of traditional medicine varies across different cultures and regions. In some cases, traditional medical systems have a well-established body of literature and documented theoretical principles and practical skills (Vedavathy, 2003). These systems are often based on extensive knowledge that has been accumulated over generations. On the other hand, certain traditional medical practises are orally transmitted from one generation to another, relying on the verbal exchange of knowledge and expertise. India has a rich history of traditional medicinal systems, namely Ayurveda, Siddha, and Unani. These medical systems are deeply rooted in ancient texts such as the Vedas and other scriptures. The concept of Ayurveda emerged and evolved between 2500 and 500 BC. Ayurvedic literature contains extensive documentation on the usage and properties of medicinal plants. The Caraka Samhita (1000 BCE–200 CE) and Susrutha Samhita (1500 BCE–1000 BCE) are among the earliest sources of information on medicinal plants. These texts provide detailed descriptions of various plant species and their medicinal properties and uses (Sharma P.V, 1992). The Caraka Samhita provides detailed descriptions of 620 plants with a total of 12,800 references (S.N Venugopal DK Ved T Ishrath Fathima, 2003). Similarly, the Susrutha Samhita contains information on 775 plants with 9,676 references (Ved D.K, 2009). The Ashtanga Samgraha, dating back to 500 CE, documents 755 medicinal plants with 20,500 references. The Ashtanga Hrudaya, written around 600 CE, also includes a wealth of knowledge on medicinal plants (D. K. V. T. I. Fathima, S. N. G. U. S. Arun. et al S.N Venugopal, 2011). After 700 CE, several additional Samhitas were written, including the Harita Samhita, Bhela Samhita, Kashyapa Samhita, Sharangadhara Samhita, and Bhavaprakasha Samhita. These texts further contributed to the knowledge and understanding of medicinal systems in India (Meulenbeld G, 2000). From the 8th century, Vyakhyas (a critical commentary written on the Samhitas) and

Nighantus (glossaries written based on medicinal plants) An exemplary work from the 17th century is the Hortus Malabaricus, which contained 794 illustrations, accompanied by descriptions of each plant in Latin and its name in four scripts (Manilal, 1984). Approximately 30 percent of the medicinal plants mentioned in classical literature, despite being extensively documented in terms of their properties and products, remain controversial when it comes to their botanical identities.

Controversy regarding botanical identity can also arise due to insufficient or inadequate morphological descriptions in the literature. In many cases, the names alone do not reveal the exact identity of the plants. However, certain plants like amla, haridra, and others that have a continuous and active tradition of use in healthcare are relatively free from controversy. To establish the identities of controversial species mentioned in the literature, it becomes necessary to consult living health traditions that possess knowledge about these plants. Close and direct engagement with these living traditions is crucial for resolving the controversies surrounding the botanical identities of such species (Thomas et al., 2020). In the context of plant identification, pharmacognosy employs various techniques and methodologies. Morphological characteristics such as the shape, size, and arrangement of plant parts, including leaves, stems, flowers, and fruits, are carefully observed and documented. Microscopic analysis is also employed to examine cellular structures and tissue organisation. Chemical analysis is another essential aspect of pharmacognosy for plant identification. Various chemical tests and assays are performed to identify the presence of specific secondary metabolites, such as alkaloids, flavonoids, terpenoids, and phenolic compounds. Modern analytical techniques like chromatography (e.g., thin-layer chromatography, high-performance liquid chromatography) and spectroscopy (e.g., infrared spectroscopy, mass spectrometry, nuclear magnetic resonance) are employed to analyse and characterise the chemical constituents of plants. Additionally, biological assays are conducted to assess the pharmacological activities and therapeutic potential of plant extracts. These assays help validate the traditional uses of plants in traditional medicine systems and provide evidence for their biological activities.

The integration of pharmacognosy in plant identification ensures a scientific and evidence-based approach. It provides valuable information about the botanical identity, chemical composition, and potential therapeutic applications of plants. This interdisciplinary field contributes to the

accurate identification, quality control, and standardisation of plant-based medicines, facilitating their safe and effective use in healthcare practises.

1.1 Ayurveda

Anuktha Dravya

यथा नानौषधं देशजानां विशेषानुसारेण यथा।

इयं तत्त्वं वाचयमानं पुक्तं स्याद्भवेत् ॥ (चरक संहिता, सूत्रस्थान २७/३३०)

Anuktha Dravya (Sharma RK., 2014)in scientific terms, refers to a substance that exhibits medicinal efficacy but lacks any explicit or implicit reference in the ancient Ayurvedic texts within a specific chronological context. It encompasses substances that have not been traditionally documented in Ayurveda but possess therapeutic potential. These substances, although novel to Ayurveda, merit inclusion in the contemporary Ayurvedic repertoire and warrant utilisation by the Vaidya community for their medicinal properties.

In Ayurveda, *S. hispida* is mainly mentioned as Vasukah and Bukah. In the commentary on "Dhanvantarinighantu' and in 'Dravyagunavijnan', Bhukah (vasukah) has been identified as *Osmanthus fragrans* Lour, while in "Bhavprakashanighantu" and 'Raja Nighantu" commentaries, no botanical name is given. Dr. K.M. Nadkarni identifies vasuka as *Indigofera enneaphylla,* which in Malayalam is called cerupullai, an entirely different plant. Rajanighantu enumerates two different kinds of vasuka, the white and the red varieties, and suggests that the white variety has a rejuvenating effect (Warrier, 1993).

Bhavaprakasha Nighantu

शिवमल्ली पाशुपत एकाष्ठीलो वको वसुः | बुकोऽनुष्णः कटुस्तिक्तः कफपित्तविषापहः |

योनिशूलतृषादाहकुष्ठशोथास्रनाशनः ||२७||

Synonyms: Ekasthila, Pasupata, Sivamalli, Vaka, and Vasu; Rasa-Katu (pungent); Tikta; Virya-Ushna; Vipaka-Katu; Effect on Tridosha; Pacifies Kapha and Pitta (Amritpal Singh, 2007)

Raja Nighantu

वसुकोऽथ वसुः शैवो वसोऽथ शिवमल्लिका | पाशुपतः शिवमतः सुरेष्टः शिवशेखरः

सितो रक्तो द्विधा प्रोक्तो वसुकौ कटुतिक्तोष्णौ पाके शीतौ च दीपनौ |

अजीर्णवातगुल्मघ्नौ श्वेतश्चैवरसायनः ||

Synonyms: Vasuko, Shivamallika, Pashupatha, Sureshta, Shivashekara, Rasa-Katu, Tiktha, Virya-Ushna There are two varieties of the plant: swetha (white) and raktha (red). (rajanighantu). There is a difference in opinion about the properties of Ayurveda according to different classical references (Tripathi Indradeo, 1998). Murivenna is a commonly used formulation in the southern part of Kerala that contains S. hispida as one of the ingredients and is widely used in Ayurveda.

1.2 Geographic distribution and taxonomy of *S. hispida*

Spermacoce hispida L is a medicinal plant that has been utilized in folk medicine in the Indian subcontinent and other tropical and subtropical continents (Sundaram & Vasanthi, 2022a).It is widely distributed in the Western Ghats of Kerala and in Maruthamalai forest, which is an extension of the Western Ghats in Tamil Nadu (Sekar & Francis, 1999). The investigation revealed that traditional healers used 85 species of plants distributed in 76 genera belonging to 41 families to treat various ailments, including *Spermacoce hispida* L, in Kancheepuram District of Tamil Nadu, India (Muthu et al., 2006).

Kerala: Kozhikode, Malappuram, Thrissur, Alappuzha, Pathanamthitta, Thiruvananthapuram, Kollam.

Odisha: Kurda district

Tamil Nadu: Alagar hills (Madurai Hills), Kolli Hills (Namakkal district), Shevaroy Hills, Kalrayan Hills, Bodamalai Hills (salem district), Pachamalai Hills (Tiruchirapalli), Chitteri Hills, Yelagiri Hills (Vellore district), Sirumalai Hills (Dindigul district), Piranmalai Hills (Sivaganga district),

Theerthamalai Hills, Dharmapuri Hills (Dharmapuri district), Gingee Hill (Villupuram district), Javadi Hills- Tiruvannamalai district figure 1.2a (Sankara rao et al ,2019.).

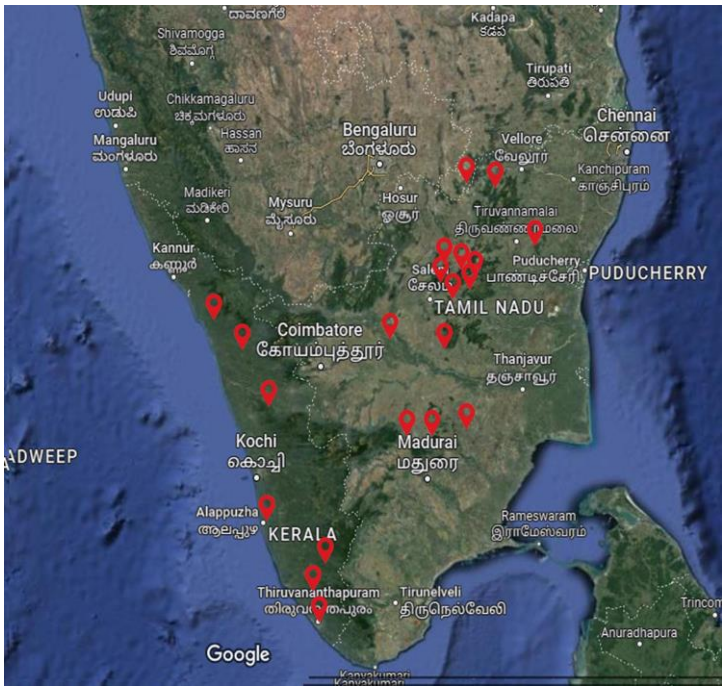


Figure 1.2a: Distribution of *S. hispida* in southern India

1.2 Plant Profile

S. hispida belongs to the family Rubiaceae, which comprises 650 genera and approximately 13,000 species (Taylor et al., 2004). In India, three Spermacoce species, *Spermacoce stricta L. f.*, *Spermacoce ocymoides Burm*. f., and *Spermacoce hispida* L., have been reported by Hooker (1881) and other early authors of Indian Floras. Sivarenjan et al. in 1987 reported that Spermacoce has nine species in India, namely, *Spermacoce confuses* Rendle, *Spermacoce podocephala* DC., *Spermacoce mauritiana* Gideon, *Spermacoce pusilla* Wall, *Spermacoce latifolia* Aubl., *Spermacoce hispida* L., *Spermacoce articularis* L., *Spermacoce ocymoides,* and *S. verticilata* Linn, wherein the latter two are restricted to South India only. Many authors have categorised Borreria and *Spermacoce*, as two different plants that belong to two distinct genera based on the morphology of their fruits, while some researchers like to merge the two taxa under the generic name *Spermacoce*. Flowering is from September to January, and Fruits are throughout the year (Sivarajan et al., 1987). The vernacular names and the

taxonomical classification of *S. hispida* are represented in Table 1.3a and Table 1.3b, Figure 1.3a.

General features of genus *Spermacoce*

Erect or diffuse herbs, branches terete to angular, leaves decussate or whorled, elliptic-Lanceolate, Chartaceous, Sub sessile, in axillary or terminal fascicles, whorls, or in sessile heads; bracteoles many, filiform. The calyx is oblong-globose; the lobes are not equal, valvate, erect, or spreading. Corolla funnel-shaped; lobes recurved, valvate, acute. Stamens between the lobes; anthers oblong Ovary 2-locular; ovule 1 per cell; (basal) axile; stigma 2-fid. Capsule globose or compressed; mericarps 2, dehiscing ventrally, rarely transversely; seeds oblong or flat, with a ventral groove; septum persistent or not (John Britto S, 2014.).

Figure:1.3a-*Spermacoce hispid* L.

Table:1.3a Taxonomic classification

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
order	Gentianales
Family	Rubiaceae
Genus	Spermacoce
Species	hispida
Author	Linnaeus (L)

Table:1.3b Vernacular names

Bengali	Madana-banta-kadu
English	Shaggy Buttonweed
Hindi	Madanaghanti
Kannada	Madanabadu, Megidda
Malayalam	Thartuvel, Kutalchurukki
Marathi	Ghanti-chi-bhaji, Gondi
Sanskrit	Madan-ghanta, Vasukah, Bukah
Tamil	Nattai-churi
Telugu	Madana-ghettu, Madanagranthi

1.3 Traditional System of Medicine

Traditional practitioners have long utilised various parts of *S. hispida* for their medicinal properties. Dried root powder is used in urinary infections, reduces internal body heat, and treats venereal diseases. Leaves for conjunctivitis and haemorrhoid gallstones. It is used as an antiseptic by the tribals of Sriharikota, an island (Sundaram and Vasanthi, 2022a).

It is used to treat stomach ailments. Flowers are applied to cure boils, eruptions, and swellings. Seeds are also used as a tonic, aphrodisiac agent. For centuries, the indigenous tribal communities residing in the forest regions of the Western Ghats in Kerala have prescribed this plant to combat signs of ageing, purify the blood, and enhance vitality. The traditional use of this plant for these purposes has been deeply rooted in their ancient practises, passed down through generations (Pushpangadan and Atal, 1984).

Siddha: In Siddha medicine It is commonly known as Nathaichuri. It has an astringent and bitter taste. The seeds can be used as a coffee substitute, and the root can be served as nannari root. It is one of the major ingredients in Nathaichuri churnam. The powdered form of this particular plant has been practised since ancient times for purposes such as weight reduction, managing dyslipidemia, and regulating blood sugar levels (KC Murugesha Mudilar, 2003.).

1.5 Pharmacognosy

Distinguishing and identifying plants and their leaves morphologically can be challenging, especially in the field and in their raw form. This difficulty arises from the resemblance of the plant and leaf characteristics to other closely related species within the same genus, such as *S. mauritiana* O. Gideon*., S. stricta* L., and *S. ocymoides* Burm (Asian J, 2011).

In all species, the leaves exhibit a dorsiventral structure, being plano-convex with a depression along the middle of the dorsal side. Detailed investigations through transverse sections have revealed that the upper and lower epidermis of the leaves are rectangular in shape. Beneath the upper epidermis, two layers of palisade parenchyma can be observed, followed by 3-5 rows of spongy parenchyma below the palisade layer. The remaining portion of the midrib is predominantly occupied by cortical parenchyma, which contains noticeable intercellular spaces.

Embedded within the midrib, a collateral vascular bundle can be observed, with the xylem positioned towards the centre and the phloem towards the periphery (Ankad G.M, 2015).

1.6 Phytochemical studies

Studies have reported that various compounds found in species of *Spermacoce* exhibit potent antioxidant activity and have significant implications for human health and disease prevention. Initial phytochemical screening of whole plant extracts of *S. hispida*, employing different solvents, revealed the presence of several bioactive constituents. These include flavonoids, phenolics, tannins, alkaloids, saponins, steroids, terpenoids, quinones, coumarins, and cardiac glycosides. Such a diverse phytochemical composition suggests the potential of *S. hispida* as a valuable source of bioactive compounds with various health benefits (Vinayak, 2013).

Through the application of analytical techniques such as HPLC, UV VIS, and FTIR, the bioactive constituents present in *S. hispida* leaves have been identified as five phenolic compounds. These compounds include kaempferol, tannic acid, epigallocatechin, quercetin, and caffeic acid (M. Shanthy, 2016).

The methanolic extract of *S. hispida* seeds, when analysed using GC-MS, exhibited a chromatogram revealing the presence of 25 peaks. The major component identified was 9, 12-Octadecadienoic acid, ethyl ester, constituting 35.58% of the total composition. Following this, 12-Octadecadienoic acid (Z, Z) and 2-hydroxy-1-(hydroxymethyl) ethyl ester were found as the second major component, accounting for 25.95% of the total composition (Dhevi R., 2015).

1.7 Pharmacological Activities

Several pharmacological studies on different standardised extracts from different parts of S. hispida have been performed both in in vitro and in vivo assay models. It shows analgesic activity and is anti-bacterial, Anti-cancer, Anti-diabetic, Anti-fungal, Antihypertensive, Anti-inflammatory, Anti-oxidant, Hepatoprotective, and Hypolipidemic (Sundaram & Vasanthi, 2022b).

A flavonoid-rich fraction derived from *Spermacoce hispida* seeds is administered to rats with high-fat diet-induced and STZ-induced diabetes. The result of the study demonstrated that the flavonoid-rich fraction extracted from *Spermacoce hispida* seeds has potential therapeutic value

in managing diabetes-related complications by modulating gene expression, improving antioxidant status, and influencing protein metabolism.

The antihyperlipidemic activity of the ethanolic extract obtained from *Spermacoce hispida* is tested in triton WR-1339-induced hyperlipidemic rats. The study aimed to explore the potential of the extract for managing high lipid levels in the bloodstream.

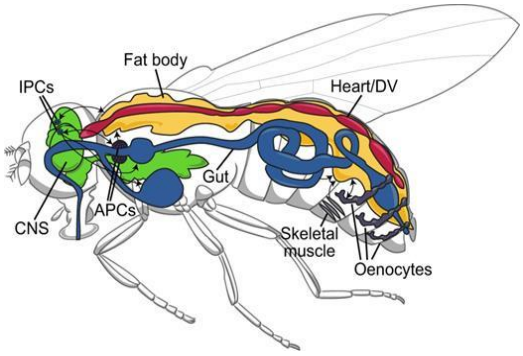
The results of the study indicated that the ethanolic extract of *Spermacoce hispida* demonstrated significant antihyperlipidemic effects in the experimental model of triton WR-1339-induced hyperlipidemia. The extract was found to effectively reduce the elevated levels of total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, and very-low-density lipoprotein (VLDL) cholesterol in the rats. Additionally, it exhibited a notable increase in high-density lipoprotein (HDL) cholesterol levels (Sivaelango. G, 2012).

The antihyperlipidemic effect of deacetyl asperulosidic acid, an iridoid glycoside isolated from the seeds of *Spermacoce hispida,* is studied, focusing on evaluating the traditional anti-obesity properties of this herb. The results of the study revealed that it effectively reduced the levels of total cholesterol, triglycerides, low-density lipoprotein (LDL) cholesterol, and very-low-density lipoprotein (VLDL) cholesterol in the experimental model. Moreover, it demonstrated an increase in high-density lipoprotein (HDL) cholesterol levels. These findings suggest that DAA isolated from *S. hispida* seeds possesses potential antihyperlipidemic properties. However, additional research is required to elucidate the underlying mechanisms of action and validate the safety and efficacy of deacetyl asperulosidic acid for human use. Further studies are needed to determine the optimal dosage and long-term effects of this compound in the treatment of hyperlipidemia and obesity (Esakkimuthu et al., 2016).

Drosophila genetics has emerged as a potent alternative model organism for investigating metabolic changes associated with diseases. Due to its short life cycle, the fruit fly offers the opportunity to rapidly assess the impacts of drugs on diverse metabolic pathways. Several studies (Tennessen et al., 2014) have utilised this model to explore these effects. Additionally, the conservation of relevant biological processes and disease-related genes between humans and *Drosophila* is noteworthy, despite the flies' comparatively lower genomic complexity relative to humans (Pandey & Nichols, 2011) . The present study utilises Drosophila as a model

organism to investigate the influence of Kashaya on the total body fat of flies. To date, investigations utilising the Drosophila model organism have not been undertaken.

1.8 *Drosophila melanogaster*- Model organism



(Laura Palanker Musselman, 2018)

Obesity and metabolic diseases, have become significant global health challenges. Understanding the molecular and physiological mechanisms underlying these conditions is crucial for developing effective therapeutic interventions. Animal models are indispensable tools for investigating the complexities of obesity and metabolic diseases, and *Drosophila* has emerged as a promising model organism due to its genetic tractability, short lifespan, conserved metabolic pathways, and the genetic tools available, including the ability to perform targeted genetic manipulations, and transgenic approaches. These techniques enable researchers to selectively modulate the expression of genes involved in metabolism and obesity, allowing for the identification and characterization of novel genetic factors contributing to these diseases.

There are similarities between the metabolic processes in flies and mammals, emphasising the conservation of key metabolic pathways and regulatory networks. *Drosophila* possesses homologs of genes involved in energy homeostasis, lipid metabolism, insulin signalling, and other critical metabolic pathways found in mammals. This conservation allows researchers to study the physiological and molecular aspects of metabolic diseases in a simplified and easily manipulable system (Musselman & Kühnlein, 2018).

Flies exhibit functional counterparts to several metabolic organs and tissues found in mammals, such as the liver, pancreas, and adipose tissue. In *Drosophila*, the fat body plays a crucial role

as a storage site for energy reserves, storing lipids and glycogen, similar to how mammalian white adipose tissue and the liver function (Canavoso et al., 2001)

Triacylglycerol (TAG) is a common component found in standard laboratory diets used in fly husbandry. While the fat body is the main organ for storing triglycerides (TAGs), other tissues also maintain their own autonomous TAG pools and express specific enzymes involved in TAG synthesis (Kühnlein, 2011). Once ingested, the dietary TAG undergoes hydrolysis by digestive lipases within the midgut lumen. This hydrolysis process results in the formation of free fatty acids (FAs), glycerol, and other acylglycerol intermediates. The metabolites derived from these TAG breakdown products are subsequently absorbed by enterocytes and utilized for the synthesis of complex lipids (Sieber & Thummel, 2009). In addition, excessive dietary fatty acids are converted into triglycerides (TAGs) and stored temporarily in intracellular lipid droplets (LDs) for storage (Kühnlein, 2011).

TAG (triacylglycerol) metabolism happens mainly in three major fly organs.

1. Midgut: The midgut is the primary site for TAG digestion and lipid absorption. Dietary TAG is broken down by digestive lipases into fatty acids (FAs), which are then absorbed by enterocytes (cells lining the midgut). Enterocytes can also produce FAs through de novo synthesis. Both the FAs from digestion and de novo synthesis are converted into diacylglycerol (DAG), which can be either transported to other tissues via the haemolymph (blood-like fluid in insects) or esterified with excessive FAs to form TAG for storage. TAG stored in the midgut can be subsequently mobilised through intracellular lipolysis, converting it back into FAs and DAG.
2. Fat Body: The fat body serves as a major energy reservoir for the fly and stores bulk TAG. Similar metabolic pathways as in the midgut are involved in the synthesis, turnover, and storage of FAs, DAG, and TAG. Adipocytes within the fat body regulate lipid transport by exporting DAG to the hemolymph and importing DAG from the hemolymph.
3. Oenocytes: Oenocytes are specialised cells that exemplify lipid utilisation in flies. FAs derived from de novo lipogenesis or TAG lipolysis are converted into structural or signalling lipids. Oenocytes can also convert surplus DAG acquired from the hemolymph into TAG for storage. TAG metabolism is not restricted to these organs alone. It is a fundamental process present in all fly tissues. Other organs, such as the central nervous

system, cardiac muscle, and skeletal muscle, also play important roles in TAG homeostasis integration and may exhibit pathophysiological phenotypes in response to TAG dysregulation (Christoph Heier, 2018).

Drosophila also allows for diet-modification studies and their impact on biological systems. Diet-induced obesity can be studied in *Drosophila* (fruit flies) by feeding them high-carbohydrate or high-fat diets, similar to the way obesity is induced in humans. In the case of high-carbohydrate feeding, high-sucrose diets are commonly used, although high-glucose and high-fructose diets have also been employed. For high-fat feeding, coconut oil is the most frequently used dietary supplement, but lard and hydrogenated soybean and palm oil products have also been utilised (Laura Palanker Musselman, 2018). A high-fructose diet increases the consumption of fructose as opposed to glucose, promoting the development of a diet-induced obese phenotype.

Studies have been conducted comparing the effects of high-fructose and high-glucose diets on *Drosophila*. They found that flies fed a high-fructose diet exhibited increased body weight, fat deposition, and triglyceride levels compared to flies fed a high-glucose diet. These findings indicate that fructose consumption plays a significant role in promoting obesity in fruit flies. While investigating body functions further, it was discovered that the high-fructose diet led to an imbalance in energy homeostasis, increased oxidative stress, and impaired insulin signalling in the flies (Rovenko et al., 2015). In this aspect, the study focused on inducing diet-induced obesity in flies using a high fructose diet and studying the effect of *S. hispida* Kashaya on full body fat of flies.

2.METHODOLOGY

2.1 Pharmacognosy

2.1.1 Sample Collection

Fresh plants of *Spermacocea hispida* used in the present study were collected from Chunangad (Latitude 10° 78’ N, Longitude 76° 40’ E) in Palakkad district, Kerala. Plant Specimens were identified, and herbarium samples (voucher numbers 126432 and 1264433) are deposited in the department of Pharmacognosy at the FRLH herbarium and raw drug repository, TDU, Bangalore. The whole plant of *S. hispida* is dried in a hot air oven at 45°C, powdered, and stored in an airtight container for further studies. Some plants are planted in the herbal garden (I-AIM’s Herbal Garden and Nursery) for future reference.

2.1.1.1 Herbarium preparation

Healthy whole plants of *S. hispida* with flowers are used for the herbarium preparation. Plant collection details are recorded. The whole plant is dipped in a solution of rectified spirit to prevent infections. Pressed specimens are placed in a well-ventilated drying cabinet. The size of the herbarium sheet used is 28 x 42 cm. Seeds are attached separately in a pouch in the herbarium sheet. Herbariums are stored in a cabinet at a maintained temperature to ensure long preservation.

2.1.2 Macroscopic characters

Fresh plants of *S. hispida* are used for macroscopic study. Morphological features of the plant parts are observed and photographs are taken. Macroscopic photographs are taken using the Nikon SMZ1000.

2.1.3 Microscopic characters

Anatomical studies were carried out to understand the tissue, Specialised structure, and biochemical components in the stem, root, and leaf. Fresh samples were taken, cut into 2.3 cm small pieces, and fixed with Formalin acetic acid (FAA). Thin sections were taken, stained with safranin, and mounted on a clean microslide with 1% glycerol. The sections were observed, and photographs were taken using an Olympus CX 33 light microscope. Figure: 2.1.3a.

Figure:2.1.3a-Olympus CX 33 light microscope.

2.1.3.1 Histochemical Studies

Freehand sections of leaf, stem, and root were taken, and detailed identification characters were noted with staining procedures (Krishnamurthy K.V., 1988). Histochemical studies were carried out for Lignin and polyphenols (TBO method), starch (iodine-potassium-potassium iodide), Lugal's iodine, Total protein (fast green method) (Ruthmann, 1970).

- Toluidine Blue O Method (TBO Method)

Thin sections are taken, stained in aqueous TBO for 30 seconds, washed in water, and mounted in 1% glycerin. Bluish green colour shows the presence of lignin, and greenish blue colour shows the presence of polyphenols.

- Iodine-potassium-iodide Method (Lugal's solution)

Sections are placed in iodine-potassium iodide and mounted in glycerin. The basic reaction appears to be the accumulation of iodine in the centre of the helical starch molecule. Shorter molecules give red, and longer molecules give blue. Newly formed starch appears in a red-purple colour and turns to a blue-black colour.

- Tannin

The same procedure is followed for starch. The section is placed in Lugal's iodine, and after that, the section is placed in 60% sulfuric acid and immediately taken and mounted on a clean slide. The presence of tannin is seen as a yellowish-orange colour.

- Fast Green Method (Total Protein)

Sections are stained with fast green by heating gently over a Bunsen burner until the stain evaporates (30–60 seconds). Remove the stain by washing it in water and mounting it in glycerin. A positive result shows bright green.

2.1.4 Physio-chemical Parameters

Moisture analysis

Moisture content was analyzed for the coarsely powdered whole plant of *S. hispida* in three trials following the SOP of the moisture analyzer (Bhanu Prathap Singh, 2021.).

Total-ash value

A powder sample of 2 g is taken to test the ash value. The sample is taken in a silica crucible charred initially on the stove and then kept inside the muffle furnace and heated to 600°C. Kept for 3 hours. At this temperature, all the organic matter will be burned, leaving behind minerals. Remove the crucible from the furnace carefully, cool it in a desiccator to room temperature, and weigh it.

2.2 Phytochemical Analysis

2.2.1 Successive Soxhlet Extraction

Successive extraction of *Spermacoce hispida* powder is done using a series of solvents with increasing polarity (hexane, chloroform, ethyl acetate, ethanol and water). Repeated extractions are performed using 18g of the samples taken in a thimble in 200ml of solvents. Each solvent extraction is done until it turns colorless. After extraction solvents are concentrated using an open method in a hot water bath. The final weight along with the extract was subtracted from the empty dish weight to calculate the extractive value. The residues obtained were resuspended in 5ml of the respective solvents and stored in the refrigerator for further analysis.

2.2.2 Qualitative Phytochemical Screening

The phytochemical analysis of *S. hispida* involved evaluating successive extracts of the plant using established protocols to identify and quantify various phytoconstituents (Ramakrishnan et al, 1994), (Evans, 1997),(Kokate CK, 1999).

- Phenols and Tannin

Ferric chloride test: A few drops of 5% ferric chloride solution are added to a few drops of filtrate. Dark green indicates that the test is positive.

Lead acetate: 3 mL of 10% lead acetate is added to 2 ml of filtrate. A bulky precipitate indicates that the test is positive.

Gelatin test: To filtrate, add 2 mL of a 1% solution of gelatin containing 10% sodium chloride. White precipitate indicates a positive presence.

- Flavonoids

Alkaline reagent test: 2 mL of 10% ammonia is added to 2 ml of filtrate and heated. Yellow fluorescence indicates the presence of flavonoids.

Shinoda test: 1 mL of methanol, a few fragments of magnesium ribbon, and concentrated hydrochloric acid are added to 2 ml of filtrate. Pink to crimson colour indicates the presence of flavanols.

- Alkaloids

Dragendorff's test: 1 ml of Dragendorff's reagent (potassium bismuth iodide solution) is added to 1 ml of the extract, an orange-red precipitate.

Mayer's test: 1 ml of Mayer's reagent (potassium mercuric iodide solution) to 1 ml of the extract—a whitish or cream-coloured precipitate.

Wagner's test: 2 ml of Wagner's reagent (iodine in potassium iodide) is added to 1 ml of the extract, a reddish-brown precipitate.

- Glycosides

Bontrager’s test: To 2 mL of filtrate, 3 mL of chloroform are added and shaken. The chloroform layer was separated, and an equal amount of 10% ammonia was added to it. The pink colour indicates the presence of anthraquinone glycosides.

Lugal’s test: one drop of pyridine and 1 ml of sodium nitroprusside were added to 2 ml of filtrate. This mixture was made alkaline using 10% sodium hydroxide. Pink to blood-red colour indicates the presence of glycosides.

- Protein and Amino Acids

Biuret test: 1 drop of 2% copper sulphate solution is added to the filtrate, and to this, 1 mL of ethanol is added, followed by the addition of potassium hydroxide pellets. The pink colour indicates its presence.

Ninhydrin test: 2 drops of ninhydrin solution are added to 2 mL of aqueous filtrate. A characteristic purple colour indicates its presence.

- Carbohydrates

Benedict's test: 1 ml of Benedict's reagent is added to 1 ml of filtrate. The mixture is heated in a boiling water bath for 2 minutes. A characteristic colour change is obtained to indicate its presence.

Fehling’s test (Detection of reducing sugar): To the filtrate, a few drops of equal parts of Fehling’s solutions No. 1 and No. 2 were added, heated on a water bath, and observed for the formation of brick-red precipitate. In most cases, the reduction took place near the boiling point and was shown by a brick-red precipitate of cupric oxide. Reducing sugars include all monosaccharides and many disaccharides.

Bradford's test: 1 mL of Bradford's reagent is added to 1 mL of filtrate and heated on a water bath for 2 minutes. Red precipitate indicates the presence of carbohydrates.

Molisch’s test: 1 mL of Molisch's reagent is added to 2 mL of filtrate, resulting in the formation of a violet ring, indicating the presence of a particular substance.

- Phytosterols

Salkowski’s test: Extracts were treated individually with chloroform and filtered to add a few drops of concentrated sulfuric acid and shake. The golden yellow colour indicates the presence of sterols.

Tshugajeu test extracts were treated with chloroform and filtered. Acetyl chloride and a pinch of zinc chloride were added to the filtrates, kept aside for 10 minutes, and then warmed up in a water bath.

- Gum and mucilage test

Alcoholic precipitation test: 25 mL of absolute alcohol was slowly added to the extracts with constant stirring.

- Resins

Acetone-water test: 25 mL of absolute alcohol was slowly added to the extracts with constant stirring. Turbidity indicates the presence of resins.

- Saponins

Foam test: Extract is diluted with distilled water and made up to 20 mL. Suspension is shaken in a tube for a few minutes. 2 cm of foam indicates the presence of saponins.

2.2.3 HPTLC fingerprinting

Successive Soxhlet extracts of *S. hispid* (hexane, chloroform, ethyl acetate, ethanol, and water) are used for HPTLC analysis. The extracts are diluted and filtered using a membrane filter. Samples were applied at a concentration of 2 µl using the CAMAG LINOMAT 5 instrument and Hamilton syringe. The stationary phase is 10 × 10 cm silica gel 60 F 254 plate. The twin-trough

chamber is used for the development of the plate. The chamber is kept saturated in the appropriate mobile phase (Table 2.2.3a) for 20 minutes before the development of the plate. The developed plate is dried, and visualization is done on the CAMAG TLC visualizer (Figure 2.2.3a). The software used for the HPTLC is Vision CATS. Images are captured in white light, UV light 254, and UV 366.

Track Assignment

Track	Vial ID	Description	Volume	Position	Type
1	1	SH HEX	2.0 µl	N/A	Sample
2	2	SH CHL	2.0 µl	N/A	Sample
3	3	SH EA	2.0 µl	N/A	Sample
4	4	SH ETH	2.0 µl	N/A	Sample
5	5	SH H20	2.0 µl	N/A	Sample
Track Assignment notes					

A track marked with ⚠ means: the application type is overridden in some evaluation(s).

System setup:

Software	Server DESKTOP-VCHNP9C, version 3.1.21109.3
Linomat 5	S/N:100106
TLC Visualizer	S/N:170805

Chromatography

Plate layout:

Stationary phase	Merck, HPTLC Silica gel 60 F_{254}
Plate format	100 x 100 mm
Application type	User
Application	Position Y: 8.0 mm, length: 8.0 mm, width: 0.0 mm
Track	First position X: 19.2 mm, distance: 15.4 mm
Solvent front position	80 mm
Notes	

Figure:2.2.3a- CAMAG TLC visualizer

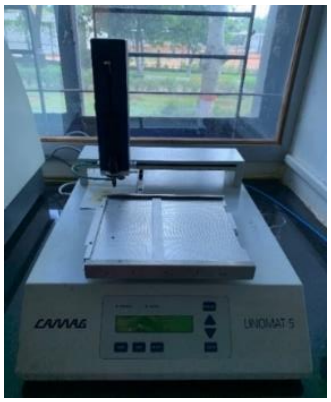


Figure 2.2.3b- CAMAG LINOMAT5

Table:2.2.3a- Mobile phase

SN	Class of compound	Mobile phase
1	General	Toluene: Ethyl acetate: Formic acid 5:4:1
2	Alkaloids	Toluene: Ethyl acetate: Methanol:25% Ammonia 30:30:15:1
3	Glycosides	Ethyl acetate: Methanol: Water 20:2.8:2
4	Polyphenols	Tetrahydrofuran: Toluene: Ethyl acetate: Water 16:8:2:1
5	Terpenes	n-Hexane: Ethyl acetate 1:1
6	Flavonoids	Ethyl acetate: Formic acid: Glacial acetic acid: water: Methanol 10:0.5:0.5:1:1

2.3 Fly Husbandry and Experimental Design

The wild-type *Drosophila melanogaster* strain, *Canton-S* (*CS*), was employed as the model organism for the experimental study. This strain is commonly used in *Drosophila* research due to its well-characterized genetic background and availability as a laboratory stock. *Drosophila melanogaster*, commonly known as fruit flies, display temperature-dependent variations in growth and developmental rates. The duration of each developmental stage is influenced by the ambient temperature. In this study, fruit flies were reared in a controlled environment maintained at a constant temperature of 25 °C within an incubator. The humidity level was regulated at 60–65%. The flies were subjected to a light and dark cycle consisting of 12 hours of light followed by 12 hours of darkness. This lighting regimen mimics the natural diurnal rhythm and ensures optimal conditions for growth and development (Michele Markstein, 2018).

2.3.1 Flipping and Sorting

Daily flipping of flies in the diet media and treatment media was conducted to ensure proper nutrient distribution and exposure to the experimental conditions. During the flipping process, the flies were gently swirled or tapped to dislodge any individuals adhering to the media surface. This technique helped prevent overcrowding and improved the availability of nutrients and treatment substances throughout the media.

Ice freezing is employed as a method during the sorting of flies to minimise potential damage. The process involves briefly exposing the flies to low temperatures by placing them on ice or in a freezer for a short period of time. This technique serves to immobilise the flies, facilitating their handling and sorting without causing significant harm. By subjecting the flies to cold temperatures, their physiological activity and mobility are temporarily reduced, allowing for controlled and safer manipulation during sorting procedures. This minimises the risk of accidental injuries or escape, ensuring accurate and efficient sorting of the flies. It is important to note that the duration of ice freezing should be carefully regulated to prevent prolonged exposure that could result in detrimental effects. The goal is to temporarily immobilise the flies without inducing long-term or irreversible damage. Once the sorting process is complete, the

flies should be transferred to suitable recovery conditions to restore their normal physiological functions.

2.3.2 Media Preparation

In the experiment, two different types of media were utilised: Diet media (control diet (Normal food) and High fructose diet) and Kashaya Media (Two concentrations of Kashaya, 5% and 10%). The media were prepared following established protocols, and their composition was carefully controlled to provide suitable nutritional support for the Drosophila melanogaster organisms during the experiment. Table 2.3.2a, Table 2.3.2a

Table: 2.3.2a Control media (100 mL)

Materials	Amount(g)	Brand
Corn flour	8	
D-glucose (Dextrose)	4	Qualigens Q15405
Sucrose	2	Qualigens Q15926
Yeast Extract Powder	5	Himedia RM027
Agar-Agar (Bacteriological grade)	0.8	Qualigens Q21185
Propionic acid	0.4	Qualigens Q26955
Benzoic acid	0.6	Qualigens SQ21665
Orthophosphoric Acid 85%	0.6	Qualigens Q29245

Table: 2.3.2b High Fructose Diet -1M Fructose (100mL)

Materials	Amount(g)	Brand
Corn flour	8	
Fructose	18	SDFCL 38433K05
Yeast Extract Powder	5	Himedia RM027
Agar-Agar (Bacteriological grade)	0.8	Qualigens Q21185
Propionic acid	0.4	Qualigens Q26955
Benzoic acid	0.6	Qualigens SQ21665
Orthophosphoric Acid 85%	0.6	Qualigens Q29245

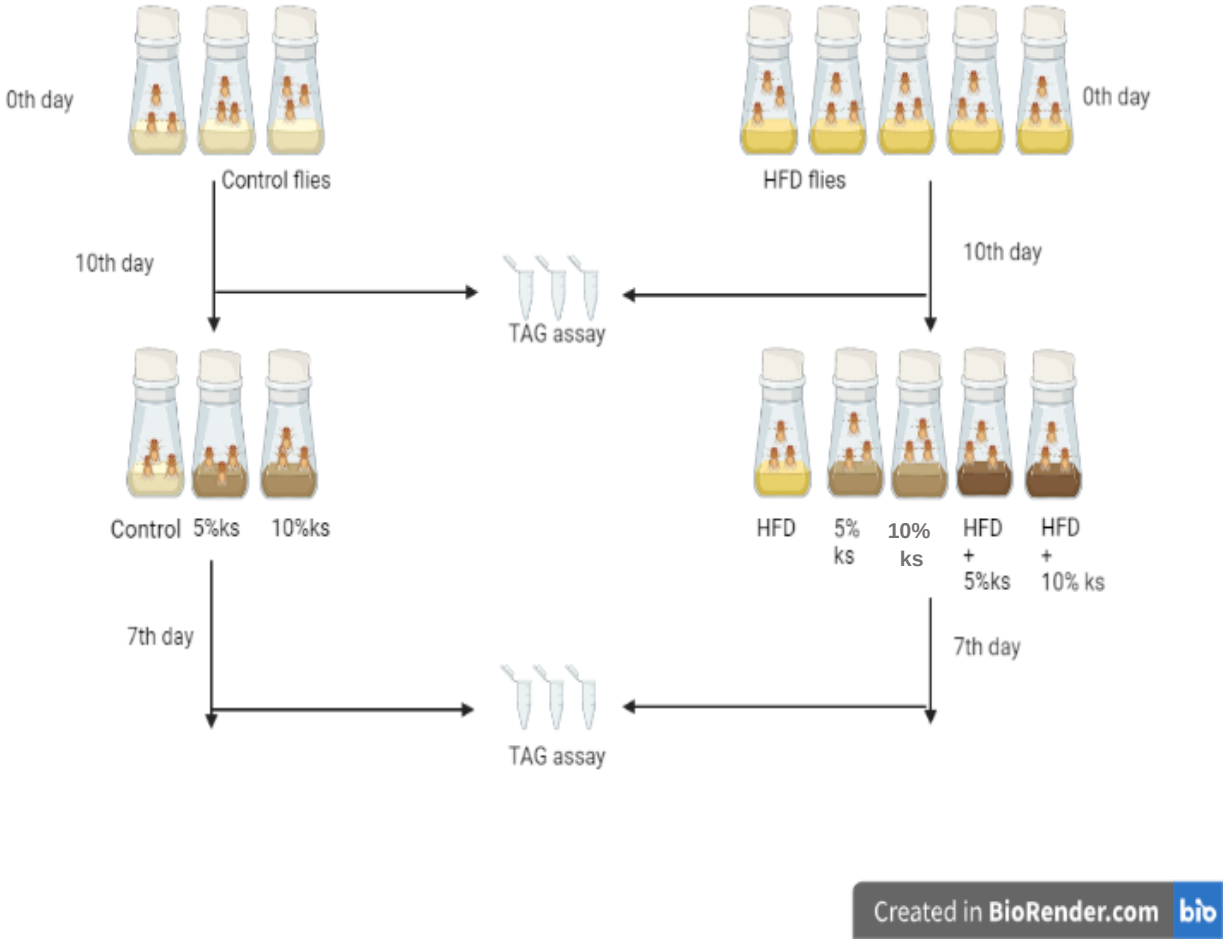
Kashaya media preparation

The powder of *Spermacoce hispida* is used for Kashaya preparation. The powder is soaked in water for 30 minutes. To 1 part of the powder, 16 times more water is added, and the mixture is then boiled on a slow flame till the water gets reduced to one-fourth of the prescribed amount (Bhatt & Deshpande, 2020). Filter the content through muslin cloth and store Kashaya at a maintained temperature. For each set of experiments, 10 g of powder is used to prepare the Kashaya. The pH of the Kashaya is maintained at 4.47.

Kashaya media is prepared in two concentrations, 5% and 10%, by adding 5 mL and 10 mL, respectively, to 100 mL of media. Two concentrations are taken to compare the effect of the Kashaya and determine the suitable dosage. For HFD Kashaya media preparation, the same method is followed, but here the Kashaya is added to HFD instead of the normal diet. HFD Kashaya is prepared in both 5% and 10% concentrations.

2.4.3 Experimental design

Newly emerged flies were used in the experiment, and we separated them into two groups, control and HFD, with each group having three bottles containing 60 flies. Feed the flies on the respective experimental diets for 10 days. On the 10^{th} day, the TAG assay is done, and 1 bottle of the control group flies is continued till the end of the experiment. 1 bottle of flies transferred to 5% and 10% Kashaya media prepared in a normal diet. One bottle of HFD flies is continued till the end of the experiment: 1 bottle to 5% and 1 bottle to 10% Kashaya prepared in normal media, and 5% and 10% prepared in HFD media. After 10 days of treatment. The TAG assay is done. The average weight of the flies is also recorded. The TAG level is normalised by using the weight of the flies. Statistical tests are performed.



2.4.4 Tag Assay

Materials

TAG standard, TAG buffer (Benesphera Triglycerides), 0.2% Tween-20, 96-well plate (Thermo), Micropipettes, heating plate, and microplate spectrophotometer (Biorad).

Method

1. Randomly select 10 flies of each gender from each group and weigh them using a microbalance.
2. Sort 3–3 flies of each gender into separate microcentrifuge tubes, and take 3 biological replicas.
3. Transfer the tube containing the flies to ice.

4. Add 200 ul of 0.2% tween-20 and homogenise it with a homogenizer, then again add 300 µL more of Tween-20 to each tube.
5. Keep the tubes in a hot water bath at 70°C for 10 minutes.
6. Centrifuge at 4000 rpm for 4 minutes.
7. Take the supernatant carefully to avoid mixing it with the pellet, and transfer it into a new Eppendorf tube.

Sample loading and TAG content determination

- Take a 96-well plate, make standards of 0, 1, 2, 3, and 4 in duplicate, and volume up to 50 µL using 0.2% tween-20 (the TAG is 2 mg/ml, so dilute it with nice water to make it 1mg/ml).
- Then take the 50-ul fly solution from Eppendorf and add it to the Elisa plate in duplicate.
- Then add 150 ul of TAG buffer to each well.
- Incubate it for 5 minutes at 37°C.
- Take the reading at 545 nm.

Calculation

TAG content (µg/mg) = TAG Concentration (µg/µL) × mean volume per fly (µL) /mean fly weight.

3.RESULT & DISCUSSION

3.1 Pharmacognosy

3.1.1 Macroscopic Characters

S. hispida is a hispid, diffuse herb that grows up to a height of 50–70 cm. The stem is green initially and purplish later as the plant ages. It is quadrangular in shape, hirsute, and sub-glabrous, with long internodes of 4.2–4.6 cm and covered with long hairs. Leaves are oppositely decussate, Elliptic-ovate 2.5–3 cm long and 1.2–1.5 cm wide, lateral nerves are 4 pairs with an attenuate base and acute-apiculate apex. Flowers are axillary; the corolla is 4, Pale purple-white, 0.8–1.1 cm long; the ovary is inferior bicarpellary. Fruits are capsule-globose, 64 mm, and seeds are 3.5 mm–4.1 mm. Chestnut brown, Oblong Septum membranous. Figure 3.1.1a, 3.1.1b, and 3.1.1c

3.1.2 Microscopic Characters

Leaf

The transverse section of *S. hispida* leaves shows a plano-convex shape. The midrib is plano-convex. It has a shallow adaxial groove and a convex abaxial surface. Trichomes are present on both abaxial and adaxial surfaces. The midrib is surrounded by cortical parenchyma cells around the vascular bundle, which are thin-walled with prominent spaces; there are 8–9 rows of cells in the abaxial region and 3–4 in the adaxial region. The vascular bundle is broadly triangular, collateral, and present towards the upper surface of the midrib. It consists of 7-8 rows of xylem elements. Lamina is dorsiventral. Epidermal cells are present on the surface. Single layer on the adaxial surface and 1-2 layers on the abaxial surface. The cells are rectangular to cylindrical in shape. Rhomboid crystals are present in the spongy parenchyma of both the midrib and lamina. Mesophyll cells are differentiated into 1-2 rows of cylindrical-shaped palisade parenchyma cells and 5–6 layers of spongy parenchyma cells. Rhomboid crystals are present in the spongy parenchyma of both the midrib and lamina. Tannin content is present in both regions. Figure:3.1.2a.

Stomata

Stomata is present on both surfaces. The abaxial surface has more stomata compared to the adaxial surface. The average stomatal index of the adaxial surface is 22.94% and that of the abaxial surface is 32.42%. Stomata are polygonal-shaped, irregular in size, and paracytic, with two subsidiary cells parallel to the guard cells. Figure:3.1.2b,

Stem

The transverse section of the stem has a quadrangular shape. Trichomes are present in the stem sections. The epidermis has a single layer with rectangular shaped cells. Stomata are visible in the epidermal region. Cortex has large oval or round-shaped parenchyma cells with polyphenol contents. 8–9 layers of cortex are present. Pericycles are thin-walled cells present around the vascular bundle. Pith is large with round parenchyma cells. Xylem is present towards the pith. Phloem elements are present along the outer end of the xylem. Xylem elements are thin-walled and narrow. Xylem rays are visible. Polyphenol content is present mainly in the pith region. Calcium oxalate crystals are common in the cortical cells. Figure:3.1.2c.

Root

The transverse section of the root has a round-oval shape. The epidermis has 2–3 layers of rectangular cells. The cortex contains 8–10 layers of parenchyma cells, which are polygonal, elongated, and oval-shaped cells. Xylem vessels are thick, and Xylem fibres are thin-walled. Polyphenol content is present mainly in the epidermis and cortical regions. Figure:3.1.2d.

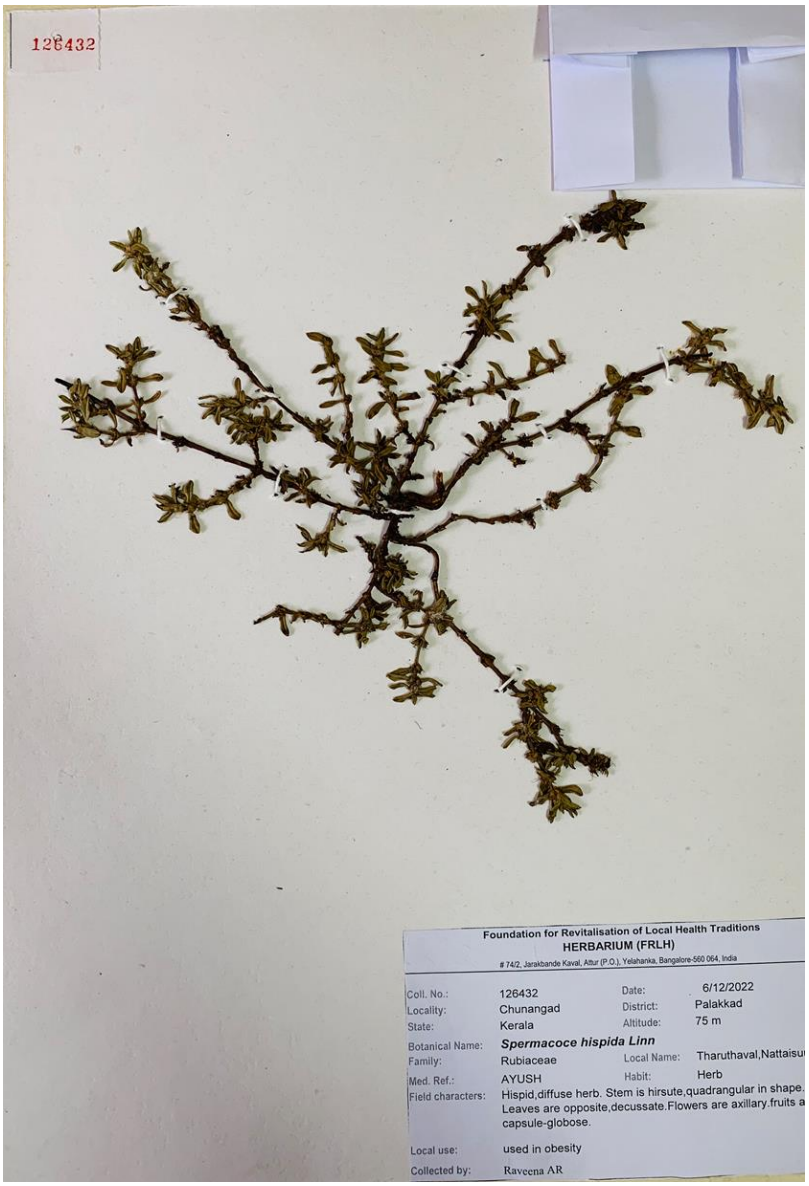
Table:3.1.2- Microscopic characters of *S. hispida*

Characters	**Leaf**	**Stem**	**Root**
Shape	Plano-convex shaped with a depression in the middle of the dorsal side.	Quadrangular shape	Round shape
Epidermis	Adaxial-Polygonal shape. Irregular size, Paracytic stomata is present, both open and closed. Stomatal index- 22.94% (Average). Stomata is less compared to the abaxial surface. Trichomes are present. Abaxial-Polygonal shaped, Irregular size. Paracytic stomata is present, both opened and closed. Stomatal index- 32.42% (Average). Stomata is more compared to the Adaxial surface. Trichomes are present.	Single layer, rectangular cells, Stomata are visible.	2-3 layer, rectangular, Parenchyma cells.
Trichomes	Present on both adaxial and abaxial surfaces.	Present	-
Cortex	Midrib is surrounded by cortical parenchyma with a collateral vascular bundle in the middle. 8-9	Large oval or rounded shape parenchyma cells	Parenchyma cells 8-10 layers, Polygonal-

	rows of cortical cells in the adaxial region and 3-4 cells in the abaxial region.	with polyphenol contents is present, 8-9 layers present	elongated-oval cells, Polyphenol content is present.
Pericycle		Thin-walled cells present around the vascular bundle.	
Vascular bundle	Broadly triangular. Midrib is plano-convex. It is surrounded by cortical parenchyma with a collateral vascular bundle in the middle. Rest of the mid rib is occupied with parenchyma cells. It is thin walled with prominent spaces.	present	present
Phloem	Periphery	Phloem elements are present along the outer end of xylem.	
Xylem	Centre	Present towards the Pith.	Xylem vessels are thick and Xylem fibers are thin walled.
Ray cells	-	Present	present
Pith	-	Irregular size, large, round cells. Polyphenol is present.	-

Crystals	Rhomboid crystal in spongy parenchyma both midrib and lamina.	Calcium oxalate crystals are common in the cortical cells.	Absent

Figure:3.1.1a- Field images of *S. hispida*. A-Location of collection. B, C habitat


126432
Foundation for Revitalisation of Local Health Traditions
HERBARIUM (FRLH)
74/2, Jarakbande Kaval, Attur (P.O.), Yelahanka, Bangalore-560 064, India
Coll. No.: 126432
Date: 6/12/2022
Locality: Chunangad
District: Palakkad
State: Kerala
Altitude: 75 m
Botanical Name: Spermacoce hispida Linn
Family: Rubiaceae
Local Name: Tharuthaval,Nattaisu
Med. Ref.: AYUSH
Habit: Herb
Field characters: Hispid,diffuse herb. Stem is hirsute,quadrangular in shape. Leaves are opposite,decussate.Flowers are axillary,fruits a capsule-globose.
Local use: used in obesity
Collected by: Raveena AR

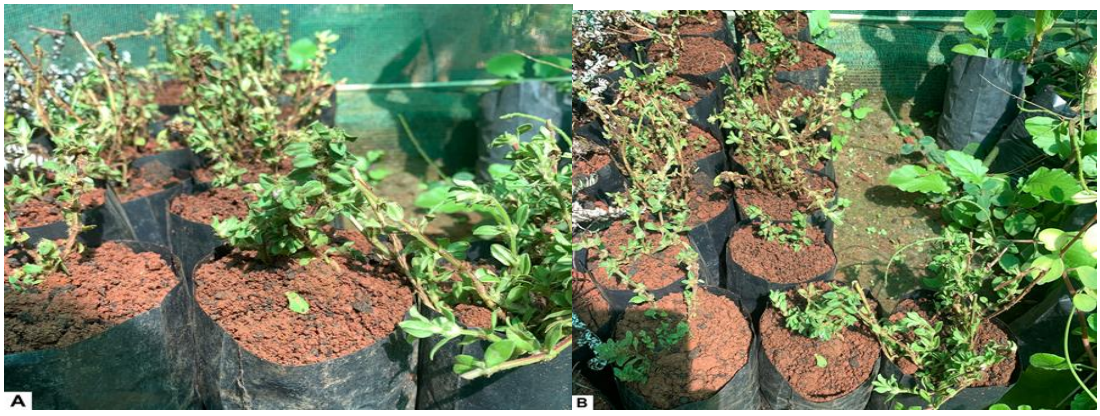


Figure: 3.1.1b- Herbarium of *S. hispida;* A, B. Freshly planted *S. hispida* plants (IAIM nursery)

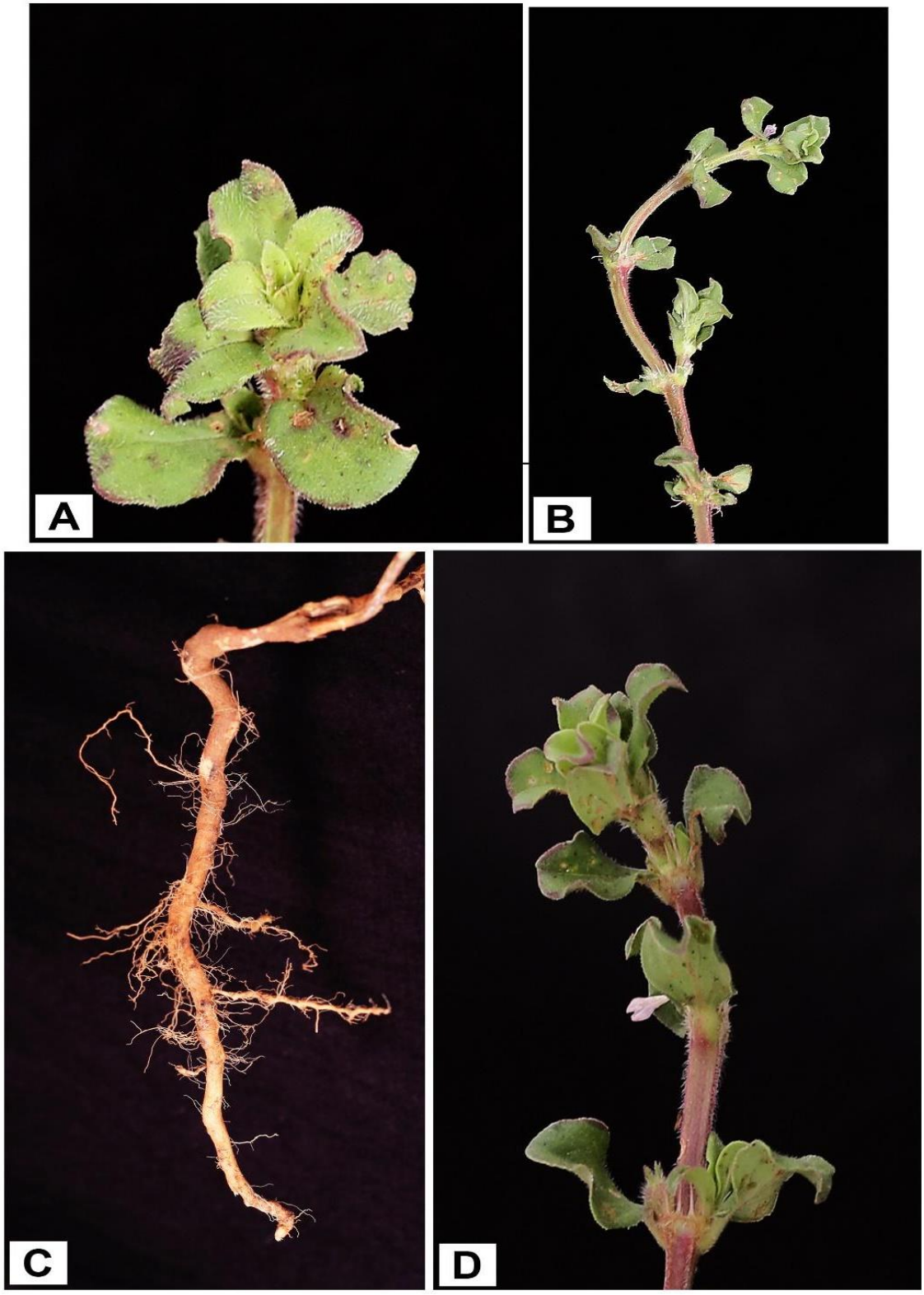


Figure:3.1.1c- Morphology pictures of *S. hispida.* A - leaves with the presence of hair; B, D-Stem with leaves, C-root, D-Stem with flower

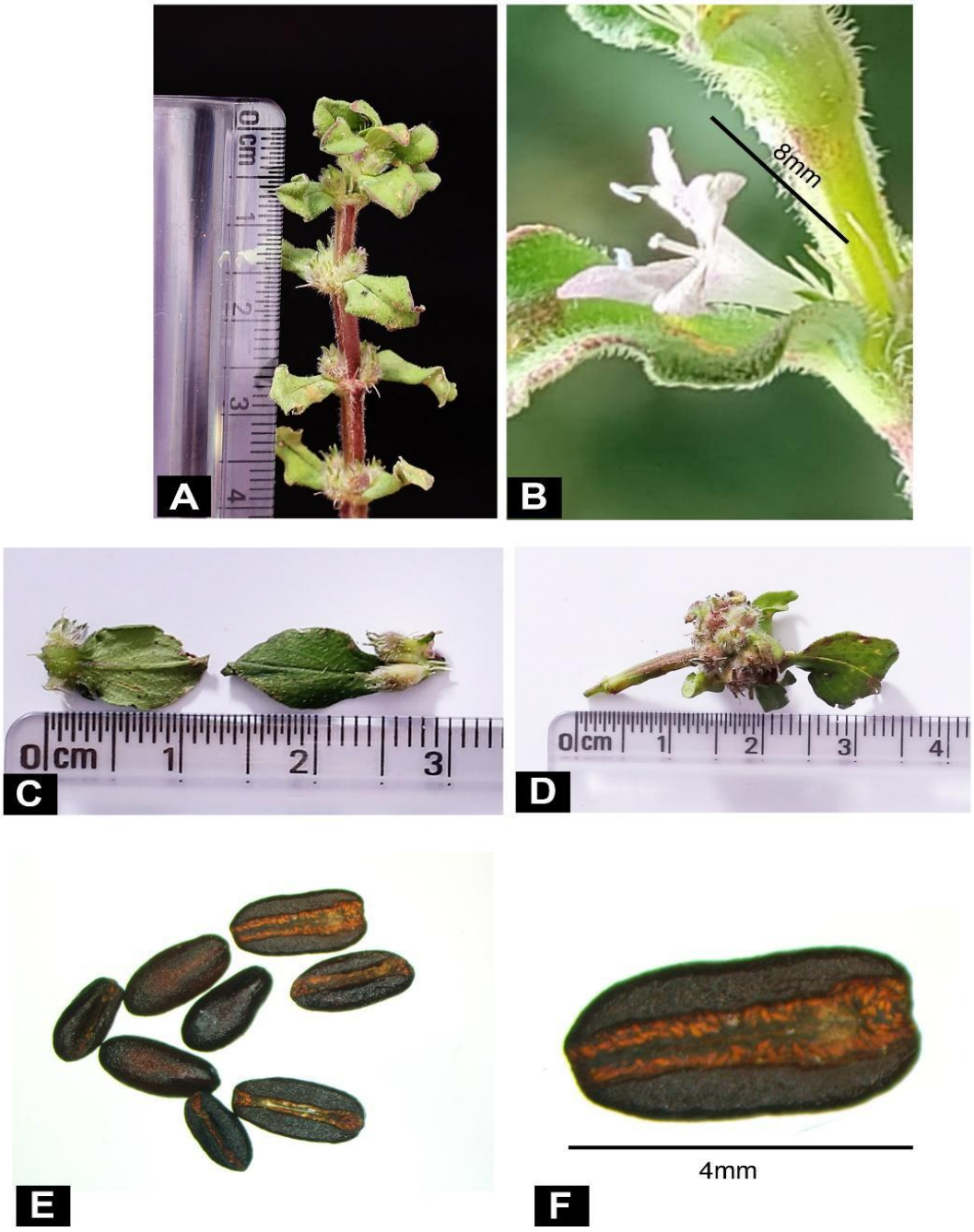


Figure:3.1.1d- A- stem showing internodes; B-Flower, C-dorsal and ventral part of the leaf; D-Fruit; E, F-Seeds.

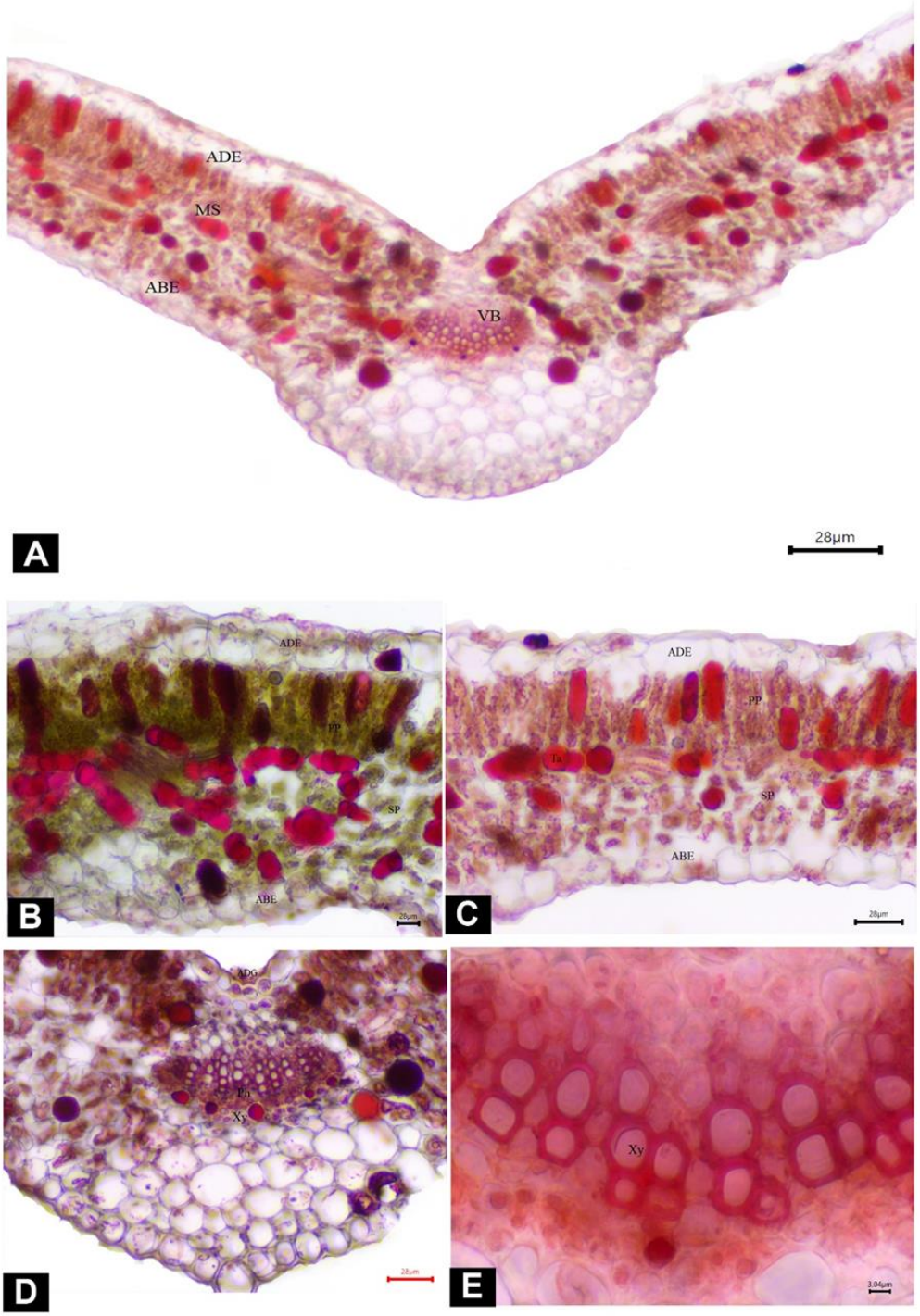


Figure:3.1.2a- Transverse section of *S. hispida* leaf. A-overview; B, C-lamina region; D, E-Mid rib region; ABE-abaxial epidermis; ADE-adaxial epidermis; Xy-Xylem; Ph-Phloem; PP-Palisade parenchyma; SP-Spongy Parenchyma; ADG-Adaxial Groove; VB-Vascular bundle

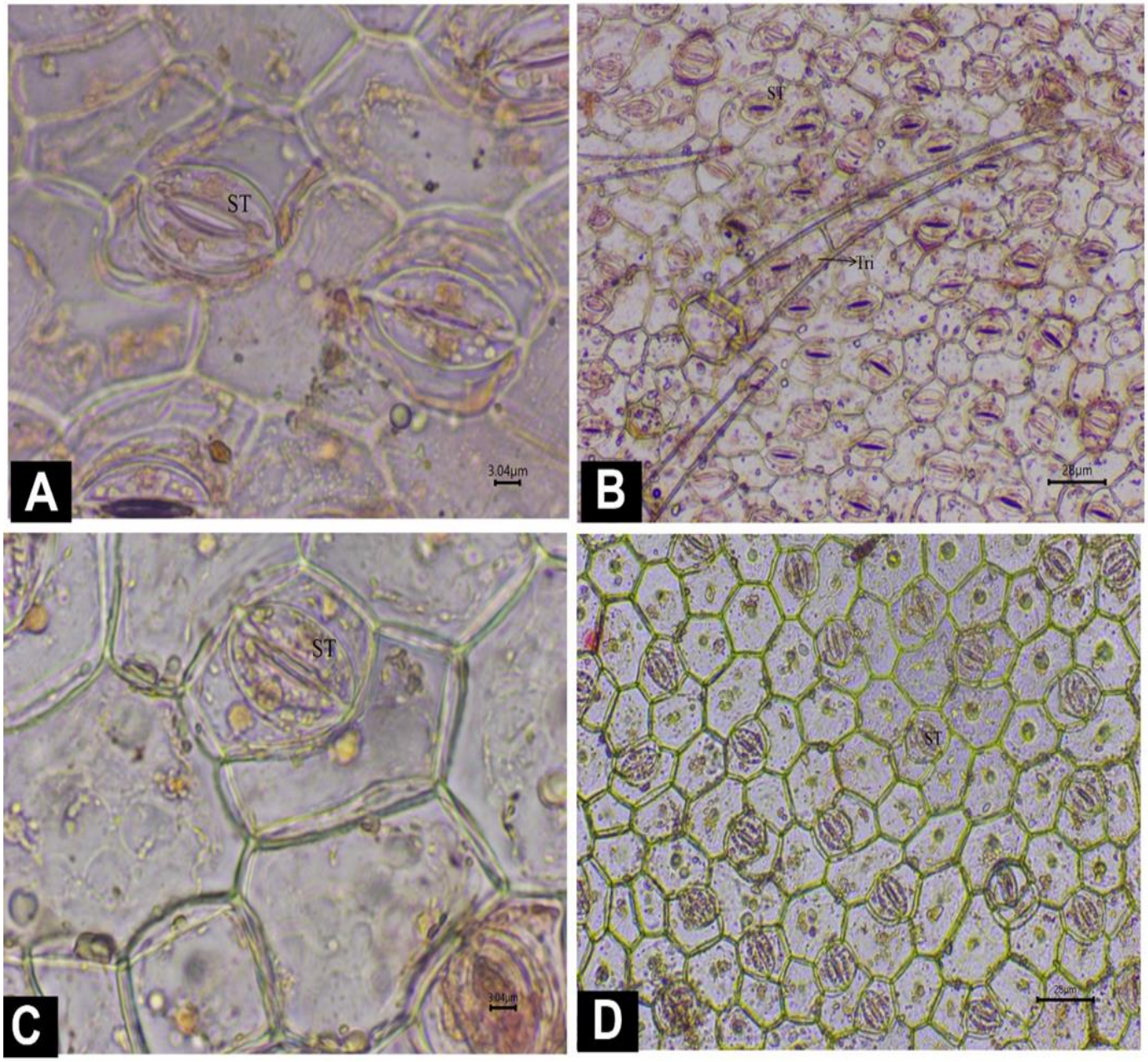


Figure:3.1.2b: Paracytic stomata. A, B-abaxial surface; C, D-adaxial surface; Tri-Trichomes; ST-Stomata.

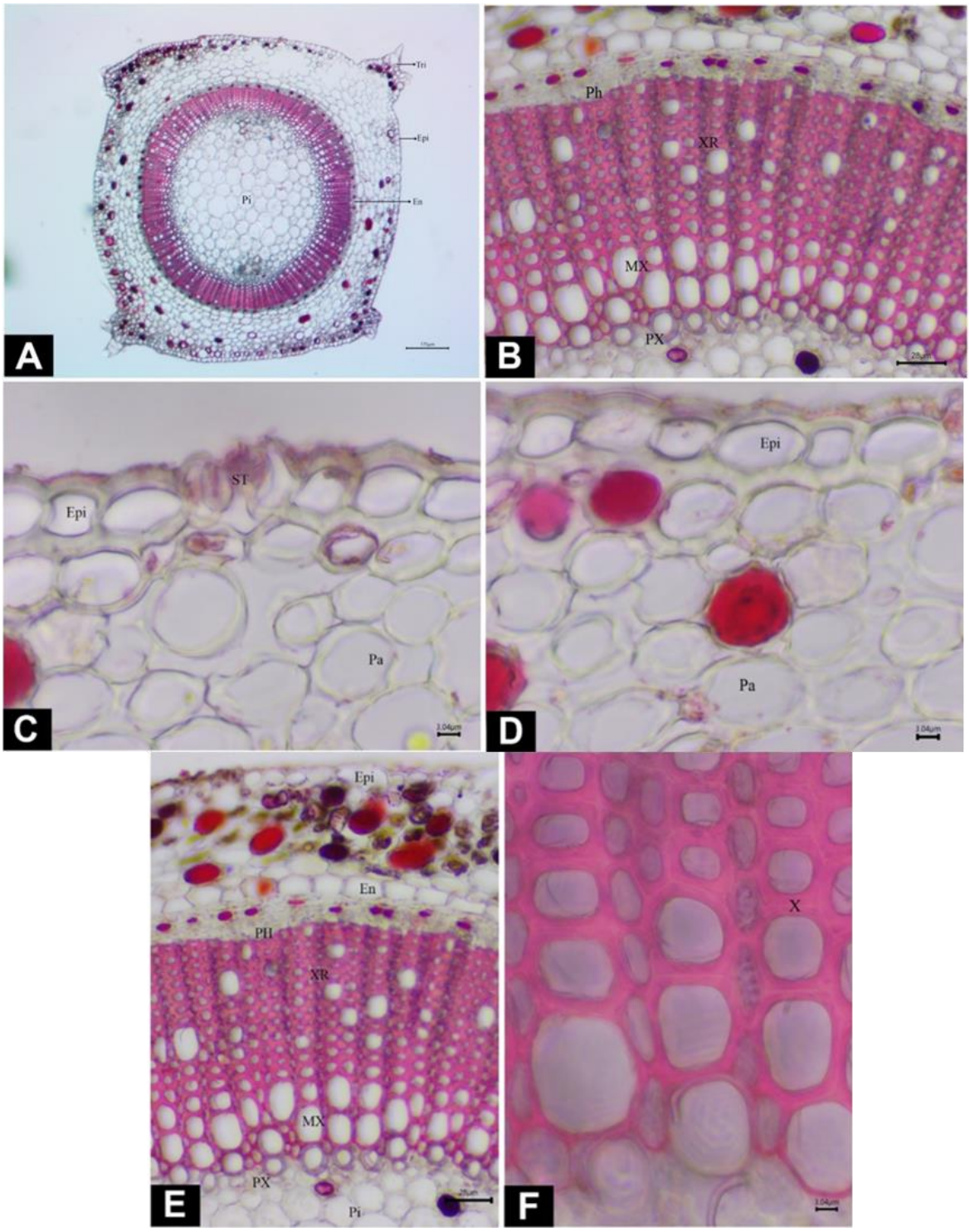


Figure:3.1.2c -TS of Stem. A-Over view of stem; B, E-vascular bundle; C-Cortical region with stomata; F-enlarged view of xylem; Pa-Parenchyma cells; MX-metaxylem; PX-protoxylem; Pi-Pith; En-Endodermis; Epi-Epidermis

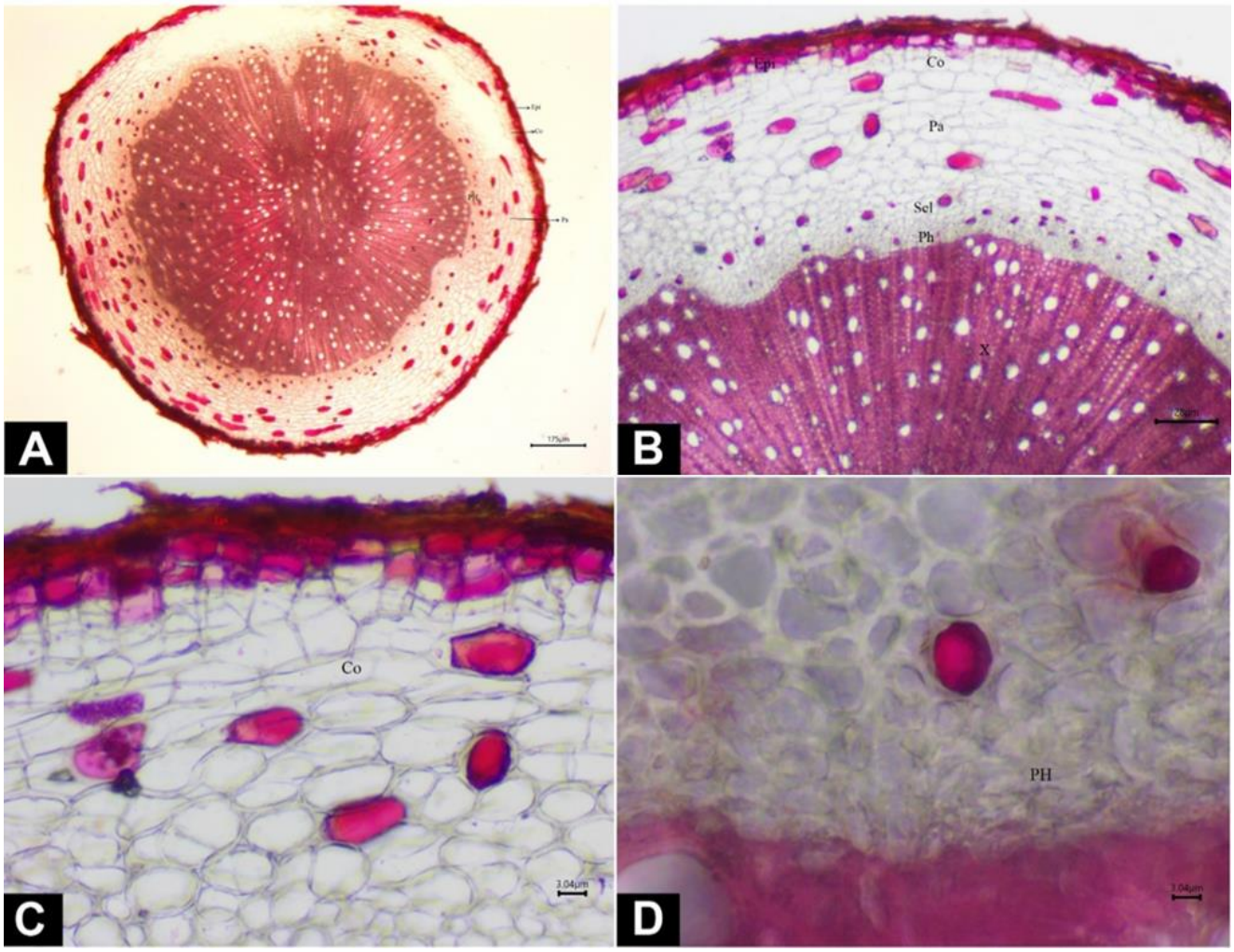


Figure:3.1.2d- TS of root. A- overview of root; B-vascular bundle; C-Cortical region; D-Phloem. Co-Cortex; Ph-Phloem; Pa-parenchyma

3.1.3 Histochemical Analysis

The histochemical analysis revealed positive results for the presence of polyphenols, lignin, starch, and protein in both stem and root sections. Polyphenols were observed in blue-green colors. Lignin appears as an orange color. Starch granules displayed a red-purple to blue-black color. Protein detection resulted in the formation of a bright green color. Table:3.1.3a.

Table:3.1.3a Histochemical analysis of *S. hispida*

Chemical Components	**Method**	**Stem**	**Root**	**Reference**
Polyphenols	TBO	Present	Present	Krishnamurthy,1984
Lignin	Lugal's Lugal's iodine+Sulphuric acid	Present	Present	Krishnamurthy,1988
Starch	Lugal's iodine	Present	Present	Krishnamurthy,1987
Protein	Fast green	Present	Present	Ruthmann 1970

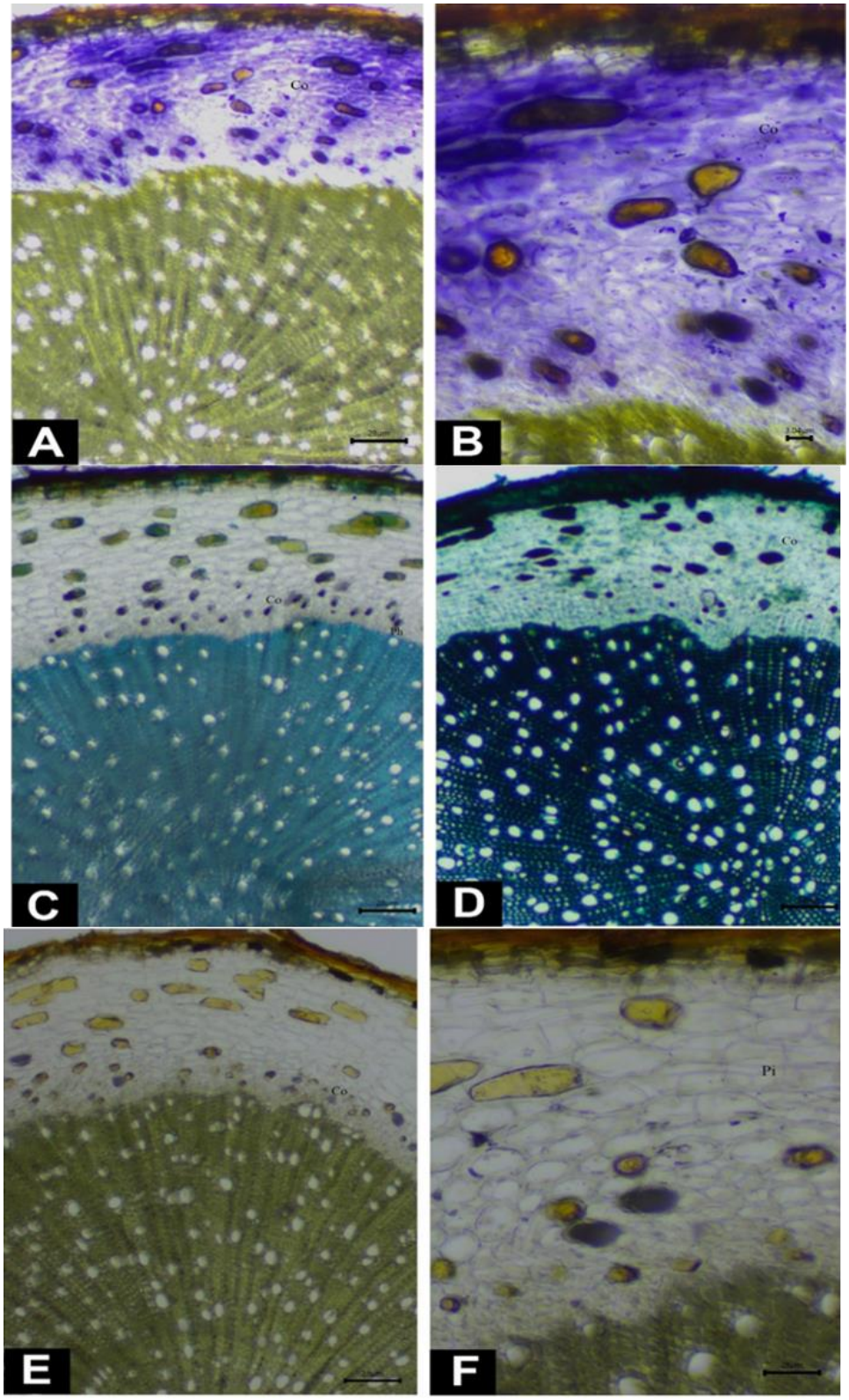


Figure:3.1.3a- Histochemical analysis of *S. hispida* root. A, Polyphenols, D-protein; E, F-starch.

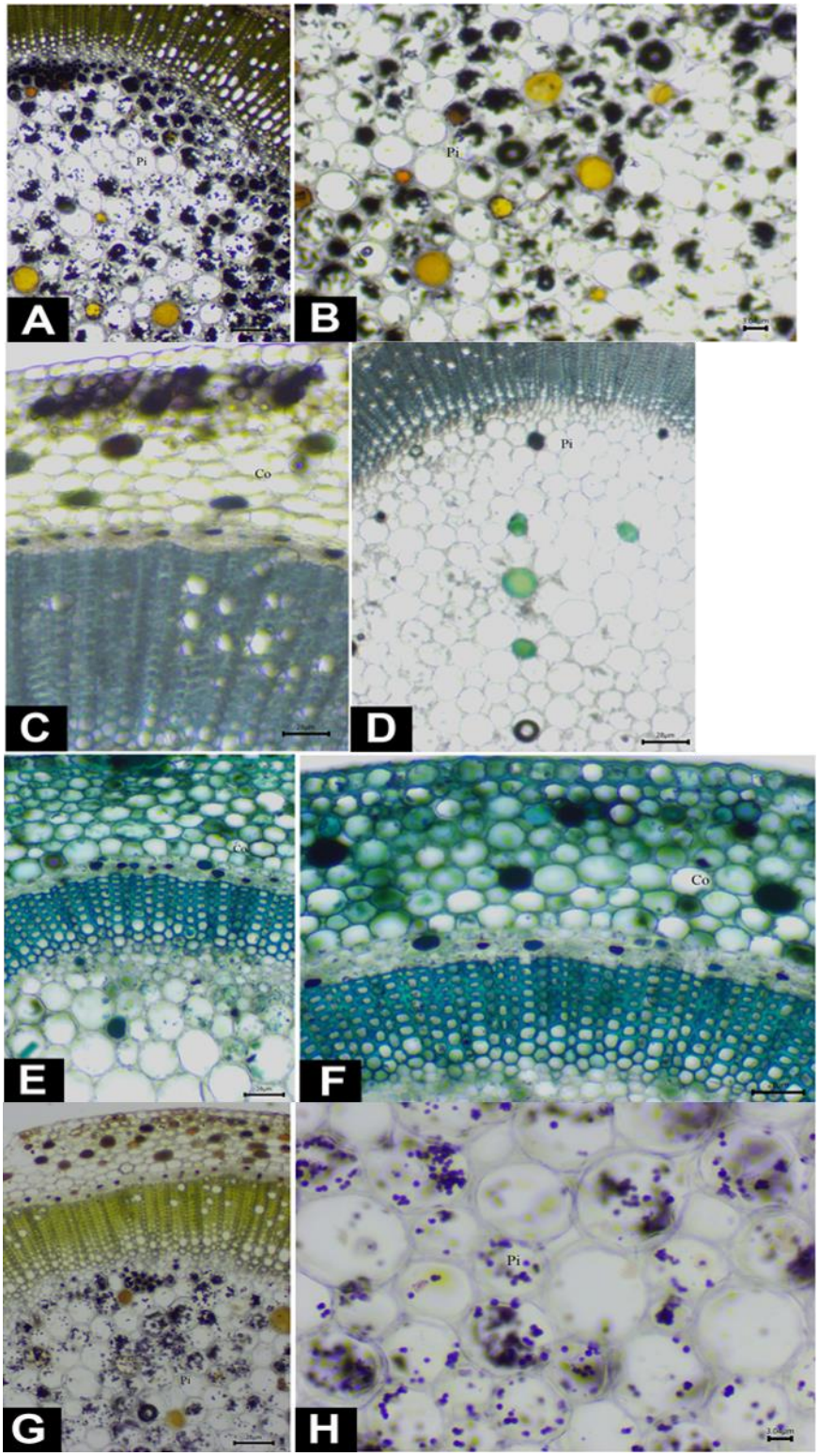


Figure:3.1.3b- Ts of *S. hispida* stem. A, B-Lignin; C, D-Polyphenols; E.F-Protein; G, H-Starch.

1.1.4 Physicochemical parameters

SN	Parameters	Result
1.	Organoleptic characteristics	
	A. Appearance	Powder
	B. Colour	Brown
	C. Odour	No characteristic odor
	D. Taste	Bitter
	E. Moisture content	6.17%
	F. Total Ash Value	6.62%

Figure:3.1.4a- *S. hispida* before drying

Figure: 3.1.4a- *S. hispida* powder

3.2 Phytochemical analysis

3.2.1 Successive Soxhlet extraction

The dried material of *S. hispida* is coarsely ground and successively extracted with a series of solvents of increasing polarity, including hexane, chloroform, ethyl acetate, methanol, and water. A total of 18 g of the sample is placed in a thimble and extracted using 200 ml of each solvent in a Soxhlet apparatus until the solvents become colorless. The extracts are then concentrated using an open drying method in a hot water bath. The resulting residues are resuspended in 5 ml of their respective solvents and stored in the refrigerator for further analysis. Figure:3.2.1a

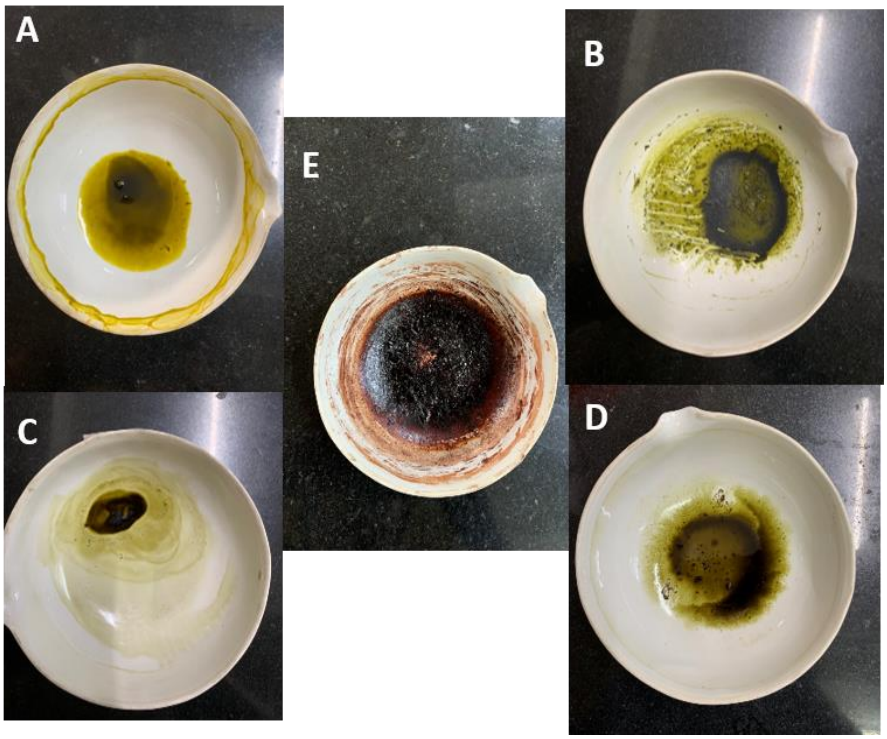


Figure:3.2.1a- Successive Soxhlet extraction of *S. hispida*. A-Hexane, B-Chloroform, C-Ethyl acetate, D-Ethanol, E-Water

3.2.2 Qualitative Phytochemical Screening

SN	Secondary metabolites and test	Successive extraction				
		Hexane	Chloroform	Ethyl Acetate	Ethanol	Water
1	Carbohydrate					
	A. Benedict's test	+	+	+	+	+
	B. Fehling's test	+	+	+	+	+
	C. Molisch test	+	+	+	+	+
	D. Barford's test	-	+	-	+	+
2.	Phenols and Tannins					
	A. Ferric chloride test	-	+	+	+	+
	B. Lead acetate	-	-	-	+	+
	C. Gelatin test	-	-	-	+	+
3.	Flavonoids					
	A. Alkaline reagent test	-	+	+	+	+
	B. Magnesium and HCl reduction (Shinoda test)	-	-	-	+	+
4.	Protein and amino acids	-	-	-	-	-
	A. Biuret test	-	-	-	-	-
	B. Ninhydrin test					
5.	Phytosterols	-	-	-	-	-
	A. Salkowski's test	-	-	-	+	+
	B. Tshugajeu test					

6.	Gum and mucilage A. Alcoholic precipitation test	-	-	-	-	-
7.	Glycosides A. Bontrager's test B. Lugal's test	- -	- -	+ -	+ -	+ -
8.	Resins A. Acetone water test	-	-	-	-	-
9.	Alkaloids A. Dragendorff's test B. Mayer's test C. Wanger's test	- - -	- - -	+ + -	+ + +	- - -
10.	Saponins A. Foam test	-	-	-	-	+

Table:3.3.2: (+) indicates positive result and (-) indicates negative result

3.2.3 HPTLC Fingerprinting

- Polyphenols

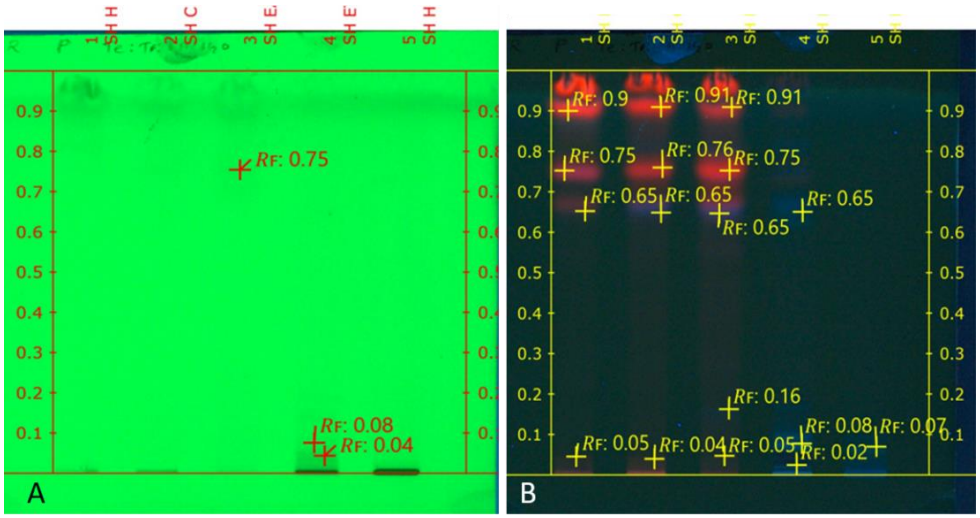


Figure 3.2.3a: The image is captured at two different wavelengths: 254 nm (A) and 366 nm (B). 1. SHH-*S. hispida* hexane; 2. SHC-*S. hispida* chloroform, 3. SHEA-S. *hispida ethyl acetate*; 4. SHE-*S. hispida* ethanol; 5. SHH20-*S. hispida* water

Extract	Solvent system	Wavelength	Rf value
Hexane	Polyphenol Tetrahydrofuran: Toluene: Ethyl acetate: Water 16:8:2:1	366 nm	0.05 0.65 0.75 0.9
Chloroform		366 nm	0.04 0.65 0.76 0.91
Ethyl acetate		254 nm 366 nm	0.75 0.04 0.16 0.65 0.75 0.91

Ethanol		254 nm 366 nm	0.04 0.08 0.002 0.08 0.65
Water		254 nm 366 nm	0.04 0.07

Polyphenol forms more bands at 366nm. At 254 nm, two bands are prominent. At 366 nm, an Rf value of 0.65 is present in all extracts. A Rf of 0.9 is present in hexane, chloroform, and ethyl acetate. Rf of 0.05 and 0.075 are present in both hexane and ethyl acetate.

- Flavonoids

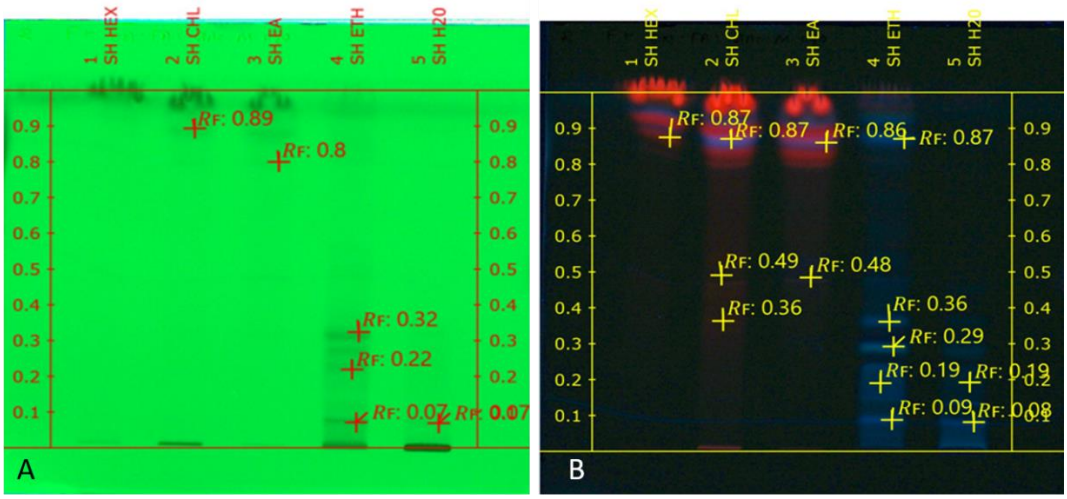


Figure 3.3.3b: The image is captured at two different wavelengths: 254 nm (A) and 366 nm (B). 1. SHH-*S. hispida* hexane; 2. SHC-*S. hispida* chloroform; 3. SHEA-S. *hispida ethyl acetate*; 4. SHE-*S. hispida* ethanol; and 5. SHH20-*S. hispida* water.

Extract	Solvent system	Wavelength	Rf values
Hexane	Flavonoids Ethyl acetate: Formic acid: Glacial acetic acid water: Methanol 10:0.5:0.5:1:1	366 nm	0.87
Chloroform		254 nm 366 nm	0.89 0.36 0.49 0.87
Ethyl acetate		254 nm 366 nm	0.8 0.48 0.86
Ethanol		254 nm 366 nm 	0.07 0.22 0.32 0.09 0.19 0.29 0.36 0.87
Water		254 nm 366 nm	0.07 0.08 0.19

At 366 nm, a Rf value of 0.87 is present in all the extracts. A Rf of 0.36 is found in both chloroform and ethanol.

➢ Terpenes

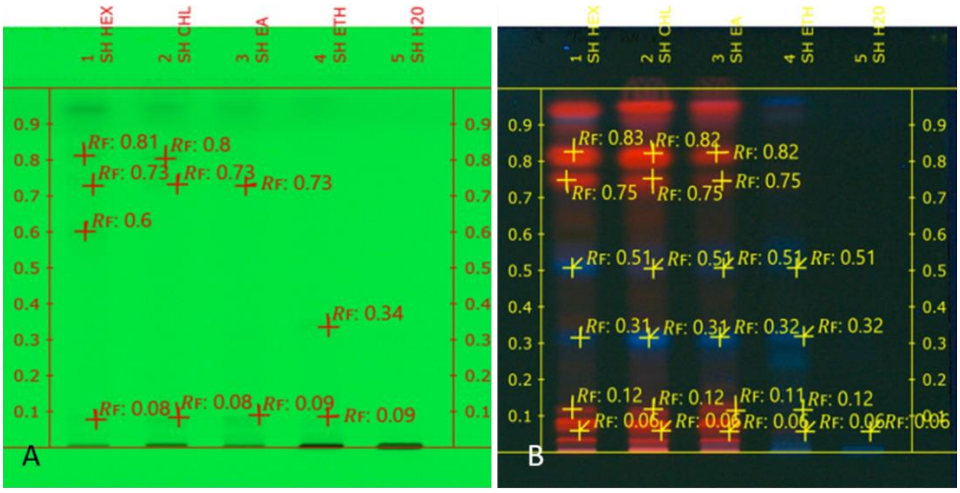


Figure 3.3.3c: The image is captured at two different wavelengths: 254 nm (A) and 366 nm (B). 1. SHH-*S. hispida* hexane; 2. SHC-*S. hispida* chloroform; 3. SHEA-S. *hispida ethyl acetate*; 4. SHE-*S. hispida* ethanol; 5. SHH20-*S. hispida* water

Extract	Solvent system	Wavelength	Rf values
Hexane		254 nm	0.08 0.6 0.73 0.81
		366 nm	0.06 0.12 0.3 0.51 0.75 0.83

Chloroform	Terpenes n-Hexane: Ethyl acetate 1:1	254 nm 366 nm	0.08 0.73 0.8 0.06 0.12 0.31 0.51 0.75 0.82
Ethyl acetate		254 nm 366 nm	0.09 0.73 0.06 0.11 0.32 0.51 0.75 0.82
Ethanol		254 nm 366 nm	0.09 0.34 0.06 0.12 0.32 0.51
Water		366 nm	0.06

At 254 nm Rf value of 0.08 in both hexane and chloroform. Rf-0.09 in both ethanol and water. Rf-0.73 in hexane, chloroform, and ethyl acetate, and Rf 0.8 in hexane and chloroform. At 366 nm, a Rf value of 0.06 is present in all extracts; a Rf value of 0.12 is present in all except water; and a Rf value of 0.75 is present in hexane, chloroform, and ethyl acetate.

➢ Glycosides

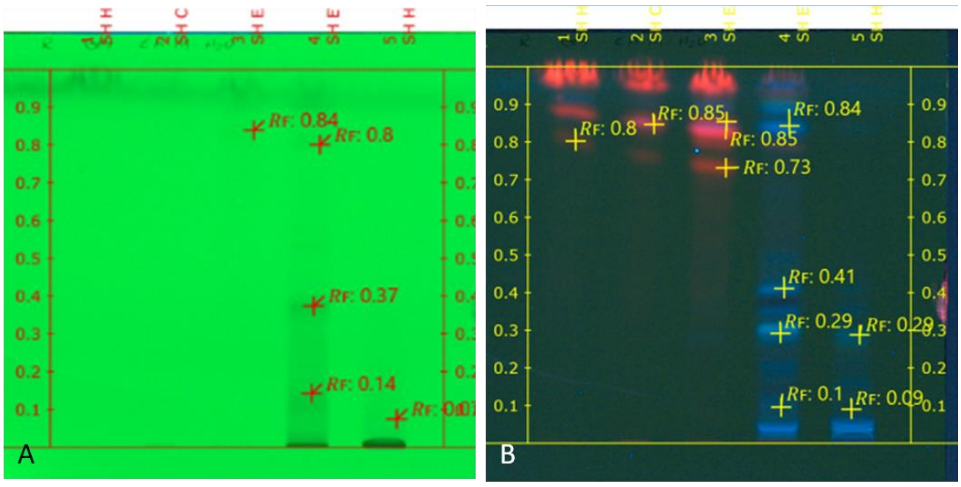


Figure 3.3.3d: The image is captured at two different wavelengths: 254 nm (A) and 366 nm (B). 1. SHH-*S. hispida* hexane; 2. SHC-*S. hispida* chloroform; 3. SHEA-S. *hispida ethyl acetate*; 4. SHE-*S. hispida* ethanol; 5. SHH20-*S. hispida* water

Extract	Solvent system	Wavelength	Rf values
Hexane	Glycosides: Ethyl acetate: Methanol: Water 20:2.8:2	366 nm	0.8
Chloroform		366 nm	0.85
Ethyl acetate		254 nm 366 nm	0.84 0.73 0.85
Ethanol		254 nm 366 nm	0.14 0.37 0.8 0.1 0.29 0.41 0.84

Water		254 nm 366 nm	0.09 0.09 0.29

At 366 nm Rf value of 0.29 is present in both ethanol and water. Rf-0.85 is present in chloroform, ethyl acetate and ethanol.

- Alkaloids

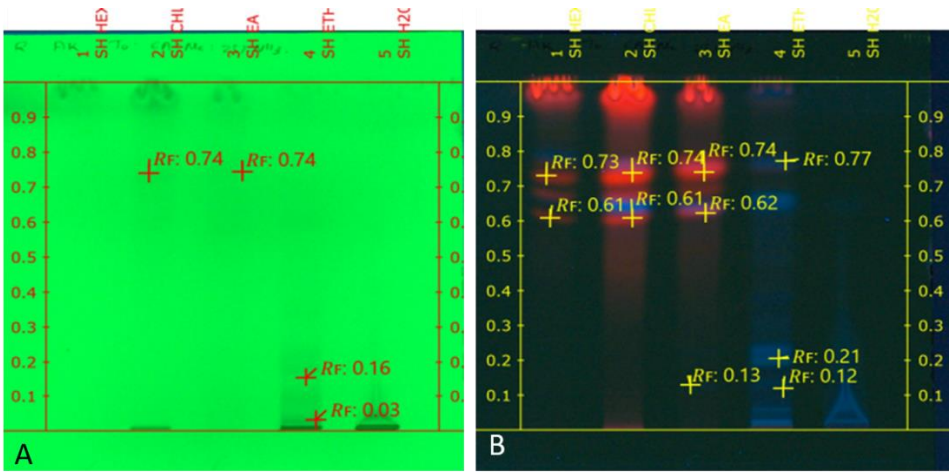


Figure 3.3.3e: The image is captured at two different wavelengths: 254 nm (A) and 366 nm (B). 1. SHH-*S. hispida* hexane; 2. SHC-*S. hispida* chloroform; 3. SHEA-S. *hispida ethyl acetate*; 4. SHE-*S. hispida* ethanol; 5. SHH20-*S. hispida* water

Extract	Solvent system	Wavelength	Rf values
Hexane	Alkaloids Toluene: Ethyl acetate:	366 nm	0.61 0.73
Chloroform		254 nm 366 nm	0.74 0.61 0.74
Ethyl acetate		254 nm 366 nm	0.74 0.62 0.74

Ethanol	Methanol:25% Ammonia 30:30:15:1	254 nm 366 nm	0.03 0.16 0.12 0.21 0.77

At 254 nm Rf value of 0.74 in chloroform and ethyl acetate. At 366 nm Rf-0.61 is present in hexane and chloroform, Rf-0.74 present in chloroform and ethyl acetate.

- General

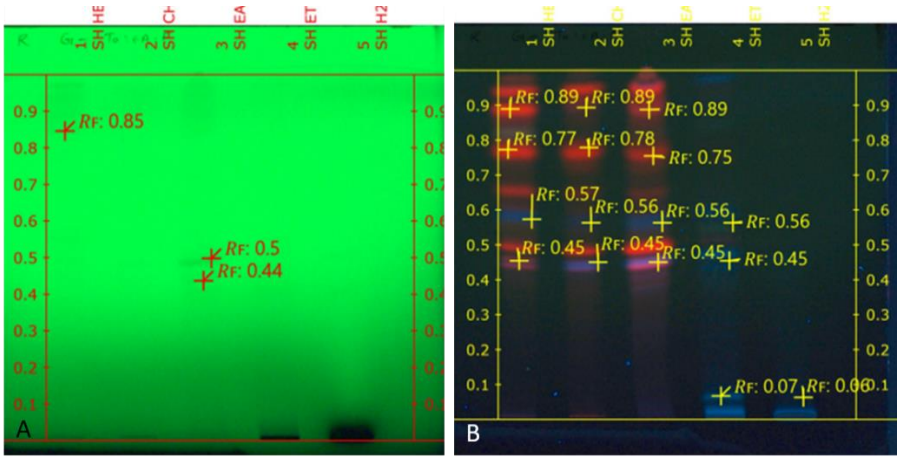


Figure 3.3.3f: The image is captured at two different wavelengths: 254 nm (A) and 366 nm (B). 1. SHH-*S. hispida* hexane; 2. SHC-*S. hispida* chloroform; 3. SHEA-S. *hispida ethyl acetate*; 4. SHE-*S. hispida* ethanol; and 5. SHH20-*S. hispida* water.

Extract	Solvent system	Wavelength	Rf values
Hexane		254 nm 366 nm	0.85 0.45 0.57 0.77 0.89

Chloroform	General: Toluene: Ethyl acetate: Formic acid 5:4:1	366 nm	0.45 0.56 0.78 0.89
Ethyl acetate		254 nm 366 nm	0.44 0.5 0.45 0.56 0.75 0.89
Ethanol		366 nm	0.07 0.45 0.56
Water		366 nm	0.06

At 366 nm Rf value of 0.45,0.56 is present in all except water, Rf- 0.89 present in hexane, chloroform and ethyl acetate.

3.3 *DROSOPHILA*-TAG ASSAY

The results of the TAG assay demonstrated a significant elevation in TAG levels in the flies subjected to a high fructose diet (HFD) compared to the control flies. Following a 10-day treatment on the Normal diet and the HFD diet, the HFD-fed flies exhibited a considerable increase in TAG levels. Subsequent transfer of the HFD-fed flies to both Normal diet media with Kashaya and HFD media with Kashaya after 7 days of treatment did not result in a significant reduction in TAG levels. In contrast, the control flies fed on 5% and 10% Kashaya media displayed a decrease in TAG levels. Notably, a trend was observed wherein the 10% Kashaya concentration showed a greater reduction in TAG levels compared to the 5% Kashaya concentration. These findings indicate that the experimental model effectively induced

alterations in TAG levels; however, the intervention of Kashaya did not yield a discernible impact on TAG levels in the HFD-fed flies.

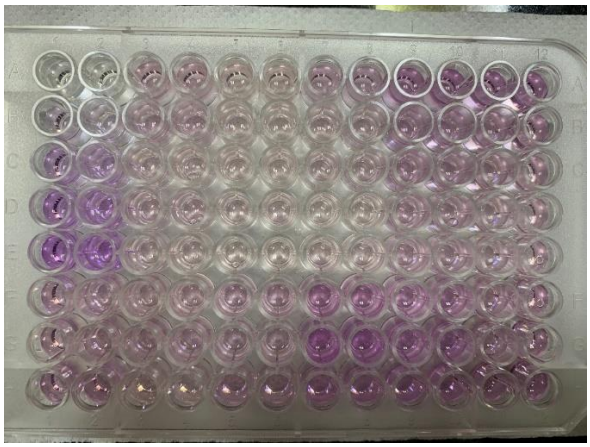


Figure:3.3a-96 well plate loaded with samples and standard for TAG assay

Pre-Treatment

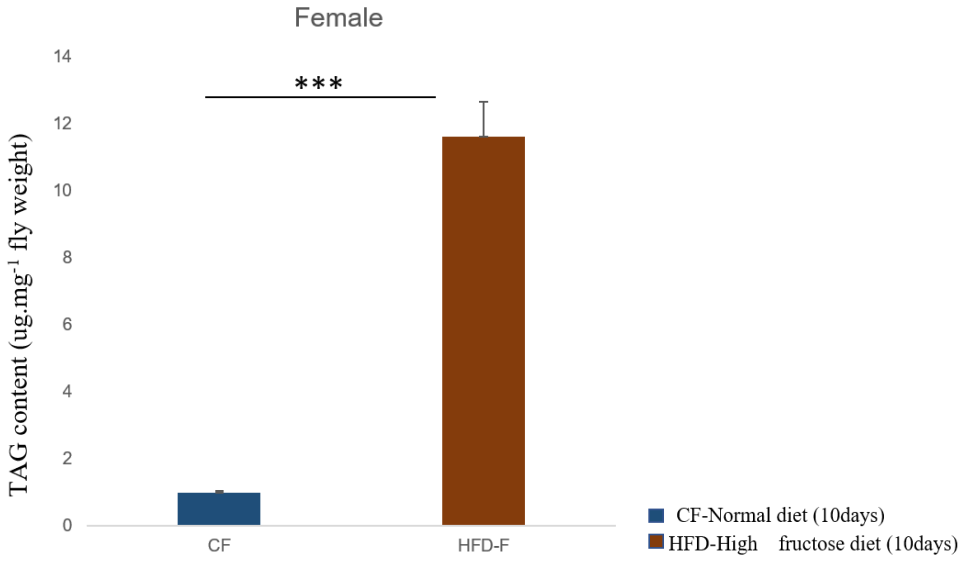


Figure:3.3b: TAG Assay-Newly emerged flies fed on Normal Diet and HFD diet for 10, Female flies; n=3; Paired t test; P value less than 0.05, is flagged with (*). If P value is less than 0.01, it is flagged with (**), If P value is less than 0.001, it is flagged with (***).

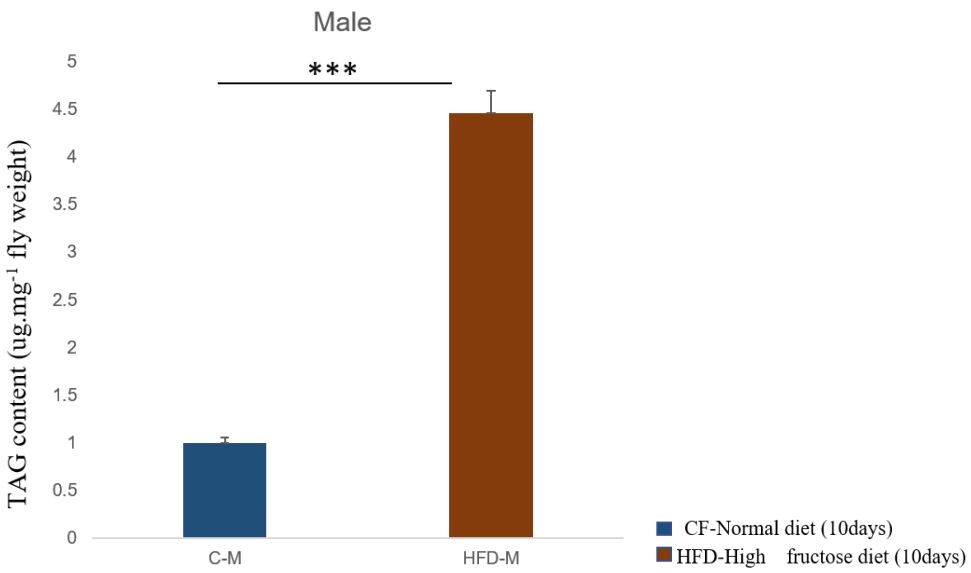


Figure:3.3c: TAG Assay-Newly emerged flies fed on Normal Diet and HFD diet for 10, Male flies; n=3; Paired t test; P value less than 0.05, is flagged with (*). If P value is less than 0.01, it is flagged with (**), If P value is less than 0.001, it is flagged with (***).

Post-Treatment

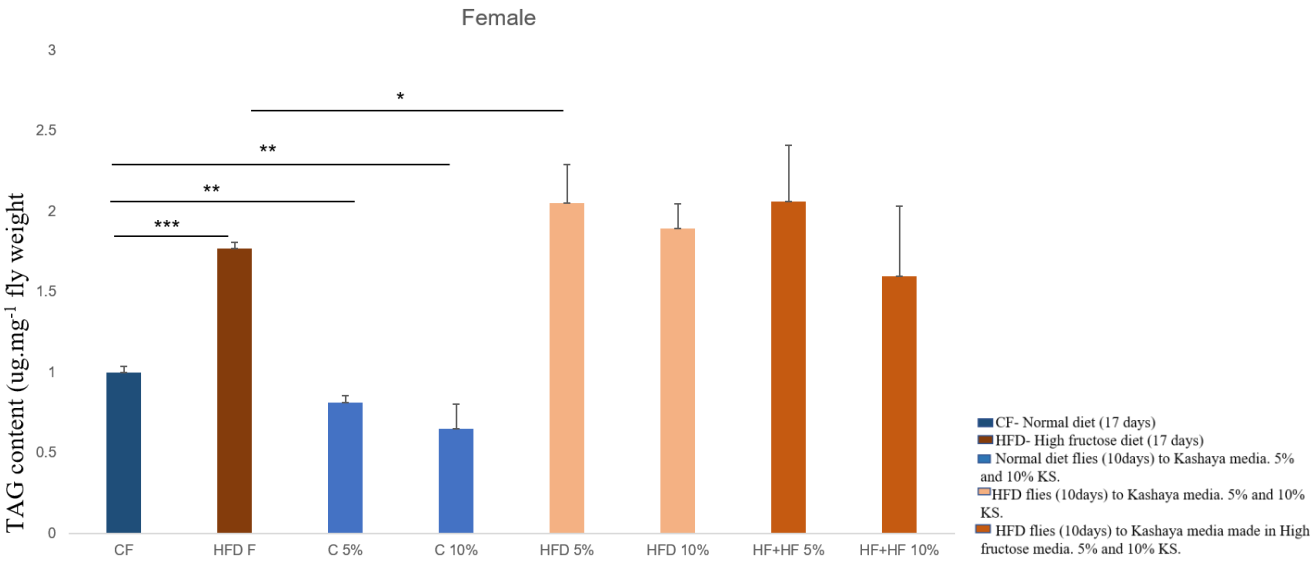


Figure:3.3d: TAG Assay- Kashaya treated Female flies for 7; n=4; Paired t test; P value less than 0.05, is flagged with (*). If P value is less than 0.01, it is flagged with (**), If P value is less than 0.001, it is flagged with (***). CF-Control flies; HFD-Hight fructose diet; C5%-5% Kashaya in control media; C10%- 10% Kashaya in control media; HFD5%-HFD flies(10days) in 5%

Kashaya; HFD10%- HFD flies(10days) in 10% Kashaya; HF+HF 5%-HFD flies (10days) in 5% Kashaya in HFD media; HF+HF 10%-HFD flies (10days) in 10% Kashaya in HFD media.

Male

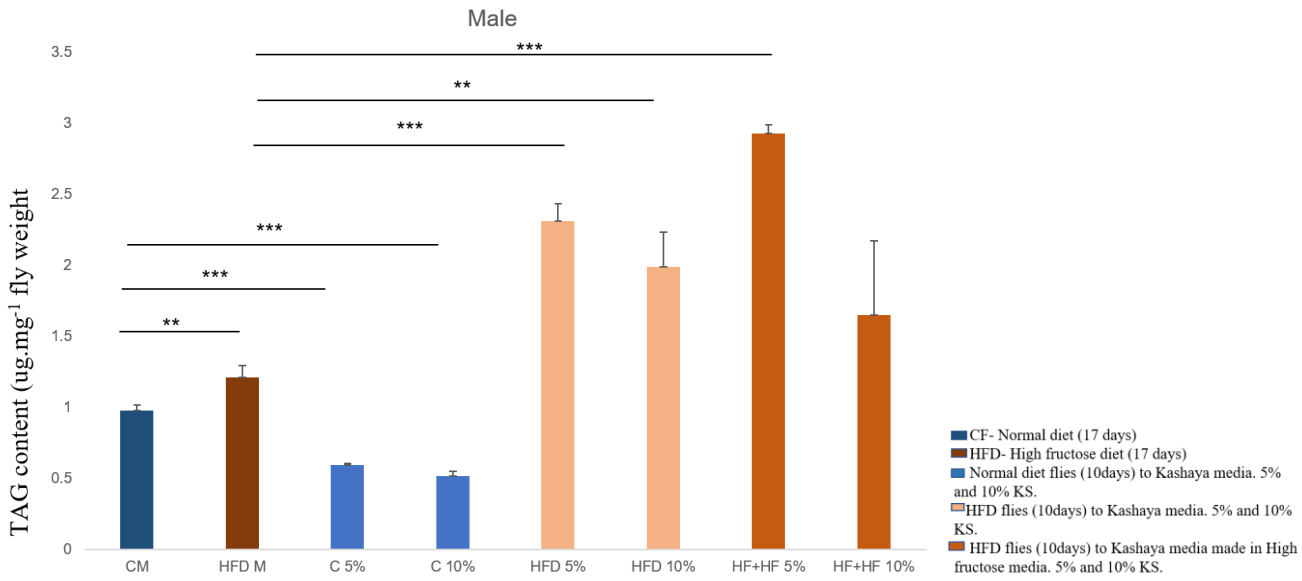


Figure:3.3e: TAG Assay- Kashaya treated Male flies for 7; n=4; Paired t test; P value less than 0.05, is flagged with (*). If P value is less than 0.01, it is flagged with (**), If P value is less than 0.001, it is flagged with (***). CF-Control flies; HFD-Hight fructose diet; C5%-5% Kashaya in control media; C10%- 10% Kashaya in control media; HFD5%-HFD flies(10days) in 5% Kashaya; HFD10%- HFD flies(10days) in 10% Kashaya; HF+HF 5%-HFD flies (10days) in 5% Kashaya in HFD media; HF+HF 10%-HFD flies (10days) in 10% Kashaya in HFD media.

4.CONCLUSION

- The Pharmacognostic evaluation of *Spermacoce hispida*, focusing on the leaf, stem, and root, along with qualitative preliminary phytochemical studies and high-performance thin-layer chromatography (HPTLC) analysis, has provided valuable insights into the plant's chemical composition. The macroscopic and microscopic studies revealed characteristic features of *S. hispida*, that are distinct from other species of the same plant. Histochemical investigations confirmed the presence of polyphenols in the root, stem, and leaf tissues. This can be used as base data for plant identification.
- The qualitative preliminary phytochemical tests and HPTLC analysis demonstrated the presence of various classes of compounds, including polyphenols, terpenes, and alkaloids, in successive Soxhlet extractions using different solvents. This analysis provides a foundation for further investigation into the chemical constituents responsible for the potential pharmacological activities of *hispida*.
- Additionally, the TAG assay conducted on flies fed a high-fructose diet (HFD) revealed a significant increase in TAG levels compared to control flies. However, treatment with Kashaya media derived from *hispida* did not result in a significant decrease in TAG levels.
- While the current findings do not support the traditional knowledge and usage of *S. hispida* for weight reduction, it is important to note that further studies are required to validate these results and confirm the potential benefits of the plant. It is crucial to conduct more comprehensive studies, including in vivo and clinical trials, to fully understand the pharmacological effects and therapeutic potential of *S. hispida* in the context of obesity. This will provide a more robust scientific foundation to either support or refute the traditional claims and help establish evidence-based guidelines for the utilisation of *S. hispida* as an anti-obesity drug.

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